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(54) Title: RAPID DETECTION AND IDENTIFICATION OF MICRO–ORGANISMS

(57) Abstract

The invention relates to the field microbiology, more specifically to the field of detection, identification and quantification or enumeration of micro–organisms. Micro–organisms, such as viruses, plasmids, bacteria, yeasts, fungi, algae, protozoa, plant or animal cells, and other prokaryotic or eukaryotic cells are in general unicellular organisms with dimensions beneath the limits of vision which thus escape easy detection. The invention provides methods and means for use in situ staining of micro–organisms comprising: a) mixing a material containing at least one micro–organism with a composition which can (partly) degrade a cell wall or cell membrane of a micro–organism thereby allowing for penetration through said wall and/or membrane of a (labelled) probe into said micro–organism, b) fixing said micro–organism with a fixative to retain its individual corpuscular character, c) reacting said probe with an antigen or nucleic acid molecule present in said micro–organism and d) detecting the presence of said probe in said micro–organism.

Title: Rapid detection and identification of micro- organisms.

The invention relates to the field microbiology, more specifically to the field of detection, identification and quantification or enumeration of micro-organisms. Micro-organisms, such as viruses, plasmids, bacteria, yeasts,

5   fungi, algae, protozoa, plant or animal cells, and other prokaryotic or eukaryotic cells are in general unicellular organisms with dimensions beneath the limits of vision which thus escape easy detection. Micro-organisms are omni-present, complex (mixed) populations can be present

10   in materials as divers as (sea)water, bodily tissues or fluids, pharmaceutical preparations, foodstuffs, industrial products, waste, soil, plants, animals, other micro-organisms, and often their (specific) presence needs to be detected or monitored. Micro-organisms for example

15   are widely used in fermentation processes, can be involved as pathogen in disease, and may be used in modern (recombinant) biotechnology; here, as elsewhere, it is essential to be able to properly identify and quantify the micro-organism(s) involved.

20   Traditionally, culturing techniques are used to establish the microbial status of a material. Culturing micro-organisms is an established way of isolating, detecting, identifying or quantifying them. Although a great number of micro-organisms can in general be

25   cultured, propagated and manipulated in the laboratory, there are also micro-organisms for which culturing methods have not been developed or for which laboratory culture may be difficult if not impossible. For viruses, and other intra-cellular organisms, in general, cell-cultures are

30   needed in which said organism grows to detect, identify or quantify the specific organism. Other micro-organisms can

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be isolated and grown in various culture media under various, often very specific, conditions.

When taking starting material in which the presence of various different micro-organisms has to be detected by

5   culturing, one generally needs to employ a diverse set of culture techniques. To be able to detect a specific organism, one has often to employ enrichment techniques, whereby the starting material is grown under conditions favouring growth of one specific (group of) organisms and

10 suppressing growth of others. Identification of organisms by culturing, however, especially in the light of the need to distinguish several (closely) related organisms (such as bacterial strains), often requires prolonged culturing under a wide variety of conditions. Furthermore, organisms

15 belonging to one specific strain may differ in that one has acquired additional genetic information, such as resistance to certain antibiotics, that is not present in another organism belonging to the same strain. Also, the micro-organism strains may differ in enzymatic activity.

20 To detect such (acquired) characteristics, yet other culture techniques are then employed.

Quantifying the number of specific organisms present in starting material via culture techniques is relatively easy when it concerns starting material containing only

25 one specific organism, e.g. in the case of monocultures. Such quantification can then be done using e.g. limited dilution tests. However, when in starting material that contains mixed populations of micro-organisms the identity and/or the relative presence or absolute numbers of

30 various organisms need to be known, culturing gets often very cumbersome and tedious, and may even be impractical or impossible for several of the organisms present in the starting material, leading to biased results.

Alternative or additive techniques to culturing for

35 the identification of micro-organisms in starting material can in principal be developed for those organisms for

which characteristics, such as specific antigenic or
genomic information is known. Using antibody or nucleic
acid probes specific for a micro-organism it is possible
to identify such organisms in various materials. In *in*
5   *situ* staining or detection, labelled probes are used that
react with cell components. Probes such as specific
antibodies, labelled directly or indirectly with reporter
molecules (chromophores or enzymes and corresponding
substrates) and used in e.g. immuno-cytochemistry,
10   generally react with antigens (especially (poly)peptides
or (glyco)proteins) that are specific for a certain micro-
organism. (Labelled) nucleic acid probes or primers
reactive with specific nucleic acids, such as genomic
(ribosomal) or episomal genes, of the micro-organism find
15   application in *in situ* hybridisation techniques, such as
FISH (fluorescent *in situ* hybridisation) or in *in situ*
amplification techniques such as *in situ* PCR (polymerase
chain reaction). Detection of the labelled antibodies or
probes or amplified sequences occurs generally by
20   microscopic observation of the material tested. Cells are
first immobilised on slides (fixed and lysed e.g. by
drying, ethanol fixation, etc.) and subsequently reacted
with antibody or nucleic acid (labelled) probes.
Alternatively, cells can be kept in suspension until after
25   the probe reaction, after which the suspension is filtered
and the cells remain immobilised on a filter disc for
analysis. Subsequent amplification of the probe signal
allows detection of the labelled cells by microscope.
Semi-automated systems, using digital image analysis and
30   image intensification (image cytometric analysis) have
been designed to help study microscopic preparations.
However, image cytometry has the disadvantage that, in
practical terms, at most some 500 to 1000 individual cells
per microscopic preparation can be analysed. In general, a
35   major drawback of above microscopic techniques is the
limited sample throughput. The long analysis time required

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limits the maximum throughput to 20 to 50 samples per day.
Furthermore, although fixation and lysis of the cells in
general suffices to immobilise antigens and nucleic acid
to the cytoskeleton or the various organelles present in
5   the cell, the cell in itself disintegrates and gets
fragmented due to the fixation and lysis. As a
consequence, those antigens and nucleic acids that are for
example present (in solution) in the cytosol will
disappear during fixation and lysis and cannot be
10   detected. As a consequence, no insight can be gained by
above microscopic techniques about (current) enzymatic
and/or mRNA activity of the micro-organisms studied. In
general, this basic problem in *in situ* staining using
signal amplification systems relates to the diffusion of
15   large-molecular weight molecules, such as enzymes,
antibodies or (strept)avidin, into whole fixed cells. The
permeabilization gets more difficult for larger molecules
since the margin between the accesibility of target
molecules and the loss of target molecules or complete
20   cell lysis becomes very narrow (Amman et al., Microbiol.
Rev. 59:143-169, 1995; Schonhuber et al, Appl. and Env.
Microbiol. 63:3268-3273, 1997). Permeabilization of cells
walls by pretreatment with lysozyme(EDTA) in general allow
a restricted set of bacteria to be investigated, whereby
25   it must be noted that lysozyme degradation often only
permeabilises a minor fraction of the tested cells
(causing heterogenous signals) and is in general only
applicable with cells fixed on microscopic slides but not
with cells in suspension.
30   Thus, techniques which allow rapid automated
detection of the identity and number of a variety of
micro-organisms present in the starting material are
needed. Furthermore, techniques are needed which rapidly
can give insight in the (enzymatic) activity of the micro-
35   organisms involved. For example, rapid techniques to
monitor the development of individual species or strains,

and/or their (current) enzymatic activity, involved in
fermentation processes comprising complex mixtures of
species would relay important information related to the
management and control of such processes. Rapid flow
5   cytometric techniques (such as FACSCAN) allow the
recognition and even sorting of whole individual cells
present in a solution; tenths of thousands of cells can be
tested in one sample, sampling of testing material can be
automated, and a multitude of samples can be tested daily.
10   Rapid flow cytometric techniques have been applied in the
detection of specific genes, nucleic acid sequences,
antigens or proteins of individual eukaryotic cells, thus
allowing rapid identification and quantification. However,
a major problem to overcome when wanting to apply flow
15   cytometry is maintaining sufficient integrity of the
individual cells which need to be tested while at the same
time making the content of cells accessible to the
necessary probes. Fragmented or disintegrated cells can
not be detected as an individual cell with flow cytometry;
20   it is essential that the corpuscular character of the cell
is maintained. There is a need for staining techniques
allowing the detection of specific traits of individual
whole cells in solution, which after all cannot be fixed
and lysed to allow free access of (labelled) probes for *in*
25   *situ* staining. Especially cells (such as most prokaryotic
cells, plant cells, and such) surrounded by a cell wall or
cell envelope have in general not been accessible to such
techniques due to the relative impenetrable character of
the cell envelope or cell wall surrounding these
30   organisms, making it impossible for (labelled) probes to
penetrate the cell and react with its specific antigen or
nucleic acid. Furthermore, cells that are only surrounded
by a cell membrane lyse easily, thereby loosing their
corpuscular character. In addition, (labelled) probes are
35   needed that can penetrate (the cell envelop) and cell
membrane and can react specifically *in situ* with antigens

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or nucleic acids contained in a (relatively) intact and
unfixed or lysed, or not immobilised cell, and substrates
are needed that can be used for *in situ* detection (e.g.
via amplification) of the bound (labelled) probe present

5　　in such cells. Although a wide body of literature exists
relating to FISH (for example EP 0497464, WO 97/05282, WO
96/36734, WO 96/34978, EP 0336412, EP 0422861, and others)
these methods are in general only applicable for a limited
number of, or even only one, species of micro-organisms,

10　　and do not allow for the detection of slowly growing or
non-growing cells. Additionally, they for example do not,
or only little allow detection of mRNA, and allow only
little or no penetration of probes into the cell. In
short, there is a need for techniques that can, while

15　　maintaining the corpuscular integrity of the majority of
the treated cells, generate sufficiently large `holes' in
cells to allow penetration of (labelled) probes and/or
reporter molecules, which need is juxtaposed to a need for
sufficiently small probes and reporter molecules that can

20　　penetrate relatively small `holes' that do not or barely
jeopardise the cell's corpuscular integrity.

The invention provides a method for use in *in situ*
staining of micro-organisms comprising: a) mixing a

25　　material containing at least one (fixed or non-fixed)
micro-organism with a composition which can (partly)
degrade a cell wall or cell membrane of a micro-organism
thereby allowing for penetration through said wall and/or
membrane of a (labelled) probe into said micro-organism,

30　　b) optionally fixing said micro-organism to further retain
its individual corpuscular character, c) reacting said
probe with an antigen or nucleic acid molecule present in
said micro-organism, and d) detecting the presence of said
probe in said micro-organism. A preferred embodiment of

35　　the method of the invention finds its application in *in
situ* staining of bacteria, yeasts or fungi, or plant

cells, such as algae, or (obligatory) intracellular
organisms, such as viruses, plasmids, and intracellular
bacteria. An example of the method provided by the
invention comprises treating a fluid containing a micro-
5   organism with a composition which at least comprises one
detergent, such as sodium taurocholate or other bile-salts
or bi-polar lipid molecules, and at least one enzyme, such
as a lipase and/or a carbohydrate degrading enzyme such as
lysozyme, finizym, or mutanolysin. Yet another example is
10   a method according to the invention wherein said
composition at least comprises a proteinase, such as
proteinase K and/or protease type XXI.

The method or permeabilization procedure represents a
controlled process by which cells become more and more
15   permeable to the probe such as a fluorophore upon prolonged
incubation. At a certain point, lysis may start to occur
resulting in diminishing fluorescence and loss of cells.
Depending on the concentration of the reactants in the
mixture and the incubation temperature, the first signs of
20   permeabilization with a mixture according to the invention
can be observed microscopically, allowing adjustment of said
incubation parameters to the conditions desired.

Additionally, said composition may comprise at least one
osmoticum, such as saccharose, sucrose, or lysine betaine, to
25   regulate osmotic pressure and/or at least one cation, such as
Ca^{++} or Li^+ (but not significant amounts of Mg^{++}). Said
composition may be optionally buffered, for example with
Tris-HCl. Furthermore, the invention provides a method for *in
situ* staining of micro-organisms wherein said micro-organism
30   is fixed, to further retain its corpuscular character, with a
fixative, for example a cross-linking agent, or selected from
the group composed of (para)formaldehyde, glutaraldehyde,
ethanol, methanol. A preferred embodiment of the invention is
a method using a nucleic acid probe, for example an RNA or
35   DNA-targeted deoxyribonucleic acid (DNA) (oligo)nucleotide
probe, a ribonucleic acid (RNA) oligonucleotide probe, or a

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peptide nucleic acid (PNA) probe. Nucleic acid probes used in
the method of the invention ("in situ probes") are provided
as well. As a consequence of the gentle, relatively non-
denaturing, method provided by the invention most nucleic

5   acid molecules in the cell are relatively little or not
denatured, thereby greatly hampering hybridisation of most
probes that otherwise would hybridise easily with stretches
of denatured nucleic acid molecules. An example of such
probes provided by the invention is given in Table 3. A

10   further embodiment of the invention entails a method wherein
said probe is labelled with a reporter molecule, for example
a reporter molecule selected from any of the group of
fluorochromes and enzymes. A preferred embodiment of the
invention is a method wherein the reporter molecule is

15   horseradish-peroxidase, preferably linked with a sulfo-SMCC
linker to said probe. A further embodiment of a method
provided by the invention is a method wherein the presence of
said (bound) probe is detected by microscopy, image
cytometry, fluorometry or flow cytometry, preferably be

20   detecting fluorescence. A preferred embodiment of the
invention is a method wherein the fluorescence of tyramine-
fluorchromes is detected. A preferred embodiment of the
invention is the use of a method of in situ staining provided
by the invention wherein the micro-organism is not

25   immobilized, for example to a slide or filter, but wherein
detection of the stained micro-organism occurs in suspension.
Also provided by the invention is a composition comprising a
cell-wall degrading reagens for use in in situ staining. Such
a cell wall degrading reagens comprises at least one

30   detergent, such as sodium taurocholate and at least one
enzyme, such as a lipase and/or aditionally comprises a
carbohydrate degrading enzyme such as lysozyme, finizym, or
mutanolysin. Additionally, said composition comprises at
least one osmoticum, such as saccharose, sucrose, or lysine

35   betaine, to regulate osmotic pressure and/or at least one
cation, such as Ca^{++} or Li^+ (but nor Mg^{++}). Said composition

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may be optionally buffered, for example with Tris-HCl. Also
provided by the invention is a nucleic acid probe linked with
horseradish-peroxidase. Said probe and horseradish-peroxidase
are preferably linked with a sulfo-SMCC linker. Also provided
5   by the invention is a diagnostic test kit for use with *in
situ* staining. Such a kit provided by the invention at least
comprises a cell wall-degrading composition and/or nucleic
acid probe provided by the invention, and can additionally at
least comprise fixative, substrate, or buffer provided by the
10   invention. The HRP-nucleic acid probe provided by the
invention is also provided for use in other tests systems
than *in situ* assays alone. Such a probe can for example be
used in enzym-linked assays , such as a dip-stick but other
formats are also possible, for the rapid detection of genes
15   or gene-products in for example lysed bacterial samples. In
such a format, the deposited reporter molecules can
additionally serve as hapten for, e.g., anti-fluorescein-AP,
antibodies via which an extra amplification step and the
detection of very low numbers of target molecules is
20   possible.
        Of basic importance to all disciplines in micro-biology
is the proper identification of the organisms at the various
taxonomic (e.g. species) level. For example, in traditional
(e.g., cheese-making) and modern (e.g. probiotics)
25   biotechnological processes, techniques to monitor the
development of individual species in complex mixed
populations are of importance to the management and control
of the process. Traditionally, this is performed by tedious
cultivation techniques. More modern methods make use of
30   ribosomal genes to identify micro-organisms (WO 91/00926).
Although such molecular (ribosomal RNA-based) methods are
suitable to identify micro-organisms directly under the
microscope without cultivation these developments are still
premature, and automated analysis is hampered by low signals
35   and limited capacity of sample throughput. Often, only 70% of
all the cells present in mixed natural populations can be

detected when universal 16S rRNA probes for fluorescence *in
situ* hybridization are applied, even when digital image
analysis and image intensification is used to collect
fluorescence emission from microscopic preparations. Although
5   image cytometric analysis is in general terms an appropriate
tool for the extraction of morphological features or
localisation parameters (e.g., as in genome-mapping of
eukaryotic cells), signal amplification is ultimately
desirable when natural mixed populations of bacteria (such as
10 those occurring in seawater) are to be investigated. Image
cytometry has the disadvantage that in practical terms, at
the most, some 500 to 1,000 individual cells per microscopic
preparation can be analysed. Furthermore, the long analysis
time required limits the maximum throughput to about 50
15 samples per day. The relatively small number of samples that
can be tested render the automation of the fluorescence *in
situ* hybridization technique practically and economically
unattainable. Flow cytometric analysis of samples would
enable large scale analysis, if technically feasible. The
20 present invention enables rapid flow cytometric acquisition
of quantitative data from probe-labelled cells and the
possibility to automate methods for *in situ* identification
based on nucleic acid probes through generation of
unequivocally high signals from such labels. Apart from the
25 need to determine the taxonomic position (scientific name) of
the micro-organism, the level of cellular (enzymatic)
activity of cells is of considerable interest. The
possibility to attach to individual cells not only a name but
also an activity in a routine procedure cq. a practical
30 protocol, will greatly advance microbiology. The detection of
certain sub-populations with changed characteristics, i.e.
that result in altered activity at the level of messenger RNA
within complex fermenting populations is of great industrial
interest. Identification of individual cells at the species
35 level via ribosomal *in situ* probing does not always yield
sufficient information on the characteristics of the mixed

population. The present invention provides a tool to measure
the potential metabolic activity or the potential level of
individual enzymes within individual cells via determination
of the concentration of various messenger RNA's. Such
5 determination optionally occurs in parallel to the procedure
used for identification of a micro-organism with (16S, 23S)
rRNA targeted DNA probes. The methods and means provided by
the invention enables the simultaneous determination of name
and activity. The rapid developments in the field of
10 genetically modified and transgenic organisms, create an
urgent need for methods to detect the modified or transgenic
genes directly in the environment. Currently, molecular
amplification, e.g. via PCR, of genes isolated from the
environment give little information on the absolute quantity
15 of such genes, their localisation, the identity or the
numbers of the host organism. Large scale field trials with
genetically modified micro-organisms can hardly be executed
due to limited availability of methods to monitor the
spreading of the organisms involved. In order to detect such
20 genes, it is most appropriate to detect the expression of the
genetic information within the organism. Currently, the most
useful method would consist of monitoring specific messenger
RNA's (mRNA's) in whole cells, i.e. without disrupting the
natural situation. Powerful signal amplification methods are
25 needed. Since the extend of enzymatic activity in organisms
is largely determined by the amount of mRNA, a method to
determine the amount of various mRNA-species in individual
cells will prove extremely valuable for a wide variety of
applications in biotechnology. Such a method has been
30 developed for mammalian cells (WO 90/11523), but has never
found wide application in microbiology due to the
inaccessibility of micro-organisms to the probes and
specifically to the size of the label used (50 kD
horseradish-peroxidase). Mammalian cells are easily
35 accessible to this label. However, bacteria, for example,
have a rigid cell envelop consisting of among others

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peptidoglycan and said method, when applied (Schonhuber et
al., Appl. and Env. Micobiol 63, 3268-3273, 1997;Lebaron et
al., Appl. and Env. Micobiol 63, 3274-3278, 1997) with
peroxidase- or biotin-labelled probes, works in general only
with well-defined target species, gives a signal
amplification of only 7-20 fold compared with conventional
fluorescence probes, is in general only applicable to cells
fixed on microscopic slides and stains only a fraction of the
cells, resulting in non-homogenous staining, again
illustrating the need for permeabilization protocols also
allowing successful in situ staining of cells in suspension.
The present invention allows for controlled degradation of
for example bacterial cell walls in combination with an
amplification technique for the measurement of rRNA in micro-
organisms thus allowing in situ staining and detection, for
example by flow cytometric detection. The present invention
provides a signal amplification of at least 30-50 fold, and
even 80 fold, as compared with conventional fluorescent
probes. The invention also provides a method to determine the
amount of messenger RNA in micro-organisms and mammalian
cells. The commercial impact of the any molecular analysis
method stands or falls with possibilities to enlarge the
scale and reduce the cost. For large scale analysis, the
ability to automate methodologies is crucial. In the ideal
case, a sample is taken, directly inserted into an instrument
and the data are printed. The system that comes closest to
this is in situ hybridisation (ISH), whereby the sample is
treated with nucleic acid probes prior to insertion into the
instrument. Quantification of the signal occurs preferably on
a cell-by-cell basis. In order to automate analysis of whole
bacterial cells, flow-cytometric detection is the most
suitable instrumentation. The signals should for example
therefore be fluorescent. Fluorescence in situ hybridisation
(FISH) in combination with flow cytometry is possible with
the invention here presented. Thus, fluorescent reporter
molecules are needed to detect genes (DNA or RNA) or

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transcripts (RNA) in cells. For any fluorescent labelling in
mixed populations to be detected by flow cytometry and for it
to be well separated from electronic background "noise-
levels", cells must contain at least some 100.000 fluorescent
5   molecules with highly fluorescent properties. This, in
contrast to "normal" fluorescent microscopic detection for
which an equivalent of some 10.000 fluorescent molecules is
needed to reach detectable levels per bacterial cell in a
mixed population. RNA's, the multi-copy transcripts of the
10 DNA, are naturally amplified within each living cell, as
such, they represent ideal targets for genetic labelling
techniques. However, each bacterial cell contains some 20.000
identical ribosomal RNA (rRNA) molecules and some 200
identical messenger RNA (mRNA) molecules. If each of these
15 molecules was tagged by one fluorochrome, signals would not
be sufficient to automate the analysis. mRNA molecules
represent the "coding" intermediates between DNA and proteins
for which the genetic code is contained in the genome. The
amount of mRNA that codes for a particular protein/enzyme
20 indicates the level of expression of the gene and gives an
impression of the potential quantity of available protein.
Therefore, the quantity of a certain species of mRNA gives an
impression of the potential enzymatic activity coded by that
messenger. Messenger RNA *in situ* hybridization (mRNA-ISH) is
25 possible as a result of the present invention. Traditional
methods to determine the activity of enzymes are based on the
formation of product or disappearance of substrate in an *in
vitro* test-system. Alternatively, antibodies or fragments of
these can be produced that specifically recognise certain
30 proteins. When such antibodies are fluorescently labelled,
the amount of fluorescence produced is proportional to the
amount of protein present. Such assays can also be performed
in *in vitro* test-systems. They can, however, not be performed
in situ in prokaryotic micro-organisms, due to the
35 inaccessibility of the bacterial cell-wall to the (large)
antibody molecule. Measurement of mRNA's, as enabled with the

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methods and means provided by the invention with *in situ*
protocols, will boost research and biotechnology in general
due to the possibility for on the spot detection of the
presence of certain species of bacteria combined with a
5   determination of their activity. This also creates the
possibility to differentiate between individual strains of
(bacterial) species. Traditional methods to determine the
composition of mixed microbial populations require
cultivation on selective and non-selective media and the
10   subsequent identification of the obtained isolates. These
methods are laborious, time-consuming and prone to
statistical and methodological error. For this reason, the
dynamics of the population composition are very difficult to
monitor. Furthermore, the detection of specific genotypes,
15   containing useful or harmful genes, can not be performed *in
situ*. In industry, specifically the food, fermentation, and
pharmaceutical industry comprising classic or modern
(recombinant) biotechnology, monitoring of for example
bacteria- or yeast-dependent fermentation processes and the
20   genetic composition and activity of the fermenting population
is essential for quality assurance for example with respect
to hygiene, product control, process control and management,
culture stability, and so on. The invention provides means
and methods to exert better control and management of such
25   cultures. For example, in modern biotechnology, heterologous
(over)expression of proteins from genes foreign to the
cultured organism is often hampered by regulation
difficulties. Although for example inducable expression of
genes governed by inducable promotors or other regulators
30   becomes more and more applied, the exact moment in time to
up- or down-regulate expression by regulating promotor
activity is hard to assess. The invention provides means and
methods to study mRNA (e.g. of foreign genes) activity in
such fermentation cultures, thereby allowing better control.
35   Environmentally, monitoring of complex mixed bacterial
populations is elemental in predicting the effectiveness of

bioremediation and gas- and wastewater-treatment. Soil
microbiologists are in need for tools that explain nutrient
regeneration potentials in terms of biomass of individual
metabolic clusters of bacteria.

5   Hybridisation probes in combination with PCR-
amplification of target nucleic acid, are often applied for
the detection of specific bacterial taxa or specific
bacterial genes in mixed populations. Currently, by using PCR
techniques the qualitative presence of a single cell against
10   a background of 10^9 non-target cells can be confirmed. As
such the technique is extremely sensitive. However, the
technique gives little or no quantitative information on
absolute cell numbers, or the distribution of the gene over
the total population. Fluorescence in situ hybridisation
15   alllows, contrary to PCR, enumeration of individual cells
that belong to a certain bacterial group, or the
demonstration of particular genes in situ. However, in
general the obtained fluorescence signals are too low to
perform high-speed automated detections and the technique is
20   too insensitive to allow the detection of single genes in
individual cells. A key factor to this problem is the fact
that oligonucleotide probes can be constructed with only a
limited number of fluorochromes (maximum of 3). When nucleic
acid probes could be combined with appropriate enzymatic in
25   stead of fluorescent labels or reporter molecules and when
these enzymatic labels would be able to synthesize hundreds
of fluorescent product molecules it would be possible to
identify single cells in situ, even if these cells were
having low amounts of target material, to detect single genes
30   in individual cells, and to detect messenger RNA species in
situ in whole cells. A prerequisite would be that these
fluorescent molecules stay in the cell. However, available
enzyme/substrate combinations produce water soluble
fluorescent molecules that diffuse out of the cell.
35   Tyramides, however, are transformed into tyramide-radicals by
horseradish peroxidase (HRP). The tendency of such radicals

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to react and bind to anything in their vicinity, thereby
remaining inside the cell, makes them ideally suited as
fluorochrome carriers. However, these can not be used because
suitable nucleic acid probes linked to horseradish-peroxidase
5   are not available. Alternative strategies, using hapten(e.g.
biotin)-labeled oligonucleotide probes which can subsequently
be detected by HRP-labelled streptavidines are routinely used
in human chromosome mapping and in situ hybridisation of
mammalian cells. However, without exception, these molecules
10 are too bulky too penetrate a (bacterial) outer cell wall
(cell envelop). With the combination of fluorescent tyramides
and HRP-labelled probes, individual genes (liberated from
fragmented cells) can be detected by FISH(WO 90/11523).
However, the probe-HRP complex is too large to freely enter
15 bacterial cells. Therefore, the invention provides specific
protocols, means and methods that are needed to permeabilize
the (bacterial) cell wall. Furthermore, the invention
provides a method to couple the HRP molecule directly to the
nucleic acid probe via a C_6-thiol linker. Such a nucleic acid
20 probe is derived from a thiolated probe linked to
horseradish-peroxidase with a sulfo-SMCC linker, or a linker
equivalent thereto. The invention herewith provides a method
for linking a nucleic acid probe to horseradish peroxidase
comprising mixing a thiol oligonucleotide with a SMCC-HRP
25 complex, incubating said mixture and purifying it by
ultrafiltration. With such a labelled probe provided by the
invention, the invention provides a probe molecule which is
small enough to penetrate a cell wall when this barrier is
properly and controllably degraded. In order to control cell
30 wall degradation, various cell wall degrading reagents and
compositions are provided by the invention that allow the
penetration of a HRP enzyme linked to an oligonucleotide by a
C6-thiol linker into bacterial cells. In order to obtain high
levels of fluorescence, tyramide-fluorochromes are
35 constructed that act as substrate for the HRP. The resulting
tyramide-fluorochrome radicals attach randomly in the cell

whereby fluorescence is attained for very long periods (up to months). The methods and means provided by the invention allow for example the direct detection of specific bacterial species (through rRNA-probing) in environmental, clinical or

5   industrial samples via microscopic observation, automated image analysis or flow cytometric methods. Also, potential enzymatic activity determined by the quantification of mRNA species can be measured *in situ* in whole cells. Also, the method and means provided by the invention allow the

10   detection of single genes within individual bacterial cells by using microscopic (image) analysis or flow cytometry. The invention allows for the use of any fluorochrome (fluorescein, rhodamine, coumarine, etc.) that can be amino-esterified. This enables the use of multiple-colors, and thus

15   the detection of various parameters simultaneously. At present, measuring mRNA in whole bacterial cells by *in situ* hybridisation to detect expression of certain genes is cumbersome, when not using the methods and means provided by the invention. The reported method uses large multiple-

20   labelled transcript probes (some 200 nucleotides long) instead of the small synthetic oligonucleotide probes and is not of great commercial interest since the production of the large transcript-probe used is very tedious and cannot be performed on a routine basis. The measurement of mRNA in

25   mammalian cells is a known and commercially available technique (ELF™, Molecular Probes, Eugene, USA). In ELF™ a fluorescent precipitate is formed that will not diffuse away. However, this methodology cannot be used for bacteria since the necessary enzyme (alkaline phosphatase) is more than

30   twice as large as peroxidase and cannot enter the bacterial cell wall without significant damage to cellular integrity and morphology. The method linking HRP to nucleic acid probes as is provided by the invention can be used to label for example peptide nucleic acid (PNA) -probes as well as

35   oligonucleotide-probes. By using PNA-probes, hybridisation can occur in the absence of NaCl which allows for

18

simultaneous use of antibodies during hybridisation.
Furthermore, the rate of hybridisation is increased several
fold by using PNA's in stead of DNA's.

5   The methods and means provided by the invention can be
used to screen large amounts of samples for the presence of
groups/genera/species/subspecies of micro-organisms by flow
cytometry and microscopy and to count the absolute number of
cells that belong to these groups. Furthermore, determination
of the absolute level of fluorescence allows for

10   quantification of ribosomal content of the cells, even when
very low levels of ribosomes are present such as in slowly
growing bacteria (oligotrophic bacteria and mycobacterium
species). ITS-targets, available in low-copy numbers, can be
targeted, which allows the detection of bacteria down to the

15   strain-level.

The methods and means provided by invention can be used
to screen large amounts of samples for the presence and
enumeration of cells that express specific mRNA's. The
quantification of the fluorescence derived from an mRNA probe

20   provided by the invention used for this purpose allows the
determination of the exact level of expression of genes
coding for e.g., toxins, metabolic enzymes, excretory
proteins, anti-microbial substances like antibiotics or
bacteriocines, lytic enzymes, (exo-)polysaccharides, membrane

25   transport proteins. Quantification of the total mRNA-pool by
using a poly-T oligonucleotide approach will enable the post-
mortem determination of the physiological state of bacteria.
This is a long-standing issue in microbiology.

The methods and means provided by the invention can be

30   used to screen large amounts of samples for the presence and
enumeration of cells that harbour specific (foreign) genes,
gene-constructs, viruses, plasmids, and so on. The
quantification of the fluorescence derived from a probe
provided by the invention used for this purpose allows for

35   the quantification of chromosomal and extra-chromosomal
genes, plasmids or gene-constructs that code for e.g.,

vaccines delivered by micro-organisms, toxins, metabolic
enzymes, excretory proteins, anti-microbial substances like
antibiotics or bacteriocines, lytic enzymes, (exo-
)polysaccharides, membrane transport proteins, food

5   preservatives, enzymes, and so on. The methods and means
provided by the invention can be used to screen large amounts
of samples for the presence of cells that harbour
bacteriophage or virus.
The HRP-nucleic acid probe provided by the invention can also

10   be used in other tests systems than in situ assays alone.
Such a probe can for example be used in enzym-linked assays ,
such as a dip-stick but other formats are also possible, for
the rapid detection of genes or gene-products in lysed
bacterial samples. In such a format, the deposited reporter

15   molecules can additionally serve as hapten for, e.g., anti-
fluorescein-AP, antibodies via which an extra amplification
step and the detection of very low numbers of target
molecules is possible.
The methods and means provided by the invention find

20   application for example as diagnostic kit in the broad field
of microbiology. A (non-extensive) list may include several
possibilities, such as: To study the effect of novel foods
(prebiotics), probiotics, and functional foods on the
intestinal microflora of both man and animal. To monitor the

25   development of mixed bacterial populations that are essential
for industrial fermentation processes in classical (cheese
and other dairy fermentation, beer, wine, etc.) as well as in
modern biotechnology (heterologous (over)expression of
proteins, etc.). To rapidly detect microbial contamination in

30   product or production tools from raw material to end product
(hygiene control). To identify and quantify the presence and
activity of micro-organisms in wastewater treatment systems.
To identify and quantify the presence and activity of micro-
organisms in bioremediation processes. To determine the

35   microbial status of (polluted) soils, waters, gasses, etc. To
determine the status of potable water, swimming water and

20

surface water with respect to pathogenic bacteria and human
health. To study the effect of antibiotics on the intestinal
microflora of both man and animal. To study the effect of
registered and novel medicines on the intestinal microflora

5   of both man and animal. To perform diagnostics/rapid
detection of pathogenic infections in man and animal
including livestock. To detect bacterial resistance to
antibiotics. To study the effect of homeopathic preparations
and reform products on the intestinal microflora of both man

10 and animal. To rapidly detect microbial contamination in
product or production tools from raw material to end product
(hygiene control). To detect specific "caries-generating"
oral streptococci for the toothpaste industry. To detect
pathogens or consortia of bacteria that are detrimental to

15 crop development, yield or quality during production,
harvest, storage and transport.

The permeabilization procedure allows for the first time
the performance of *in situ* hybridization experiments with all
of the bacteria which contain rigid polysaccharide capsules,

20 extensive peptidoglycan layers, extraordinary cross-linking
moieties in their peptidoglycan layer and/or that can prevail
in the form of impermeable spores. Cells need not be brought
to the vegetative state prior to *in situ* hybridization. The
invention allows for the first time the direct detection of

25 non-growing cells or cells obtained directly from the natural
environment by *in situ* hybridization. By combining enzymatic
labeling of oligonucleotides and methods to allow the
entering of such conjugates into the cells as well as the
formation of fluorescent precipitates, the present method

30 ensures sufficient signal amplification as to yield
fluorescence signals strong enough to allow the *in situ*
detection by fluorescence in situ hybridization of low copy
numbers of rRNA molecules. Thereby, the present invention
provides a methodology to detect, e.g., *Mycobacterium*

35 *tuberculosis* directly in sputum samples or bacteria obtained

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from soil or bacteria obtained from watersamples by *in situ* hybridization. Such cells normally contain only 50-100 ribosomes and can not be detected by conventional fluorescence *in situ* hybridization.

5   The invention allows for the first time the direct detection of messenger RNA's by *in situ* hybridization. By combining enzymatic labeling of oligonucleotides and methods to allow the entering of such conjugates into the cells as well as the formation of fluorescent precipitates, the

10   present method ensures sufficient signal amplification as to yield fluorescence signals strong enough to allow the *in situ* detection by fluorescence in situ hybridization of the naturally (very) low copy numbers of mRNA. Therefore, the present invention provides a methodology to detect, e.g.,

15   protease gene expression in Lactococcus lactis cells by *in situ* hybridization. Such cells normally contain only 50-100 mRNA molecules that can not be detected by conventional fluorescence *in situ* hybridization.

The invention allows for the first time the direct

20   detection of streptococci by *in situ* hybridization. The present method provides for a permeabilization formula which can permeabilize streptococcal cell-walls without the need of prior formaldehyde fixation. The present invention thereby provides a methodology to detect, e.g., streptococci by *in*

25   *situ* hybridization. Such cells can not be detected by conventional fluorescence *in situ* hybridization techniques. The invention allows for the first time the quantitative measurement of cells stained by *in situ* hybridization on a flow cytometer. The present method provides for a methodology

30   with which cell lysis is prevented. Due to such a prevention, post-hybridization washing of cells is now effective (no background signal) and cells can be measured quantitatively by using methods such as flow cytometry.

The invention provides for the first time an optimized

35   methodology of *in situ* hybridization staining by which cells

from natural samples, such as occur in feacal flora, can be
measured on a flow cytometer. The present method provides for
a methodology in which the pH of the buffer is adjusted such
that FISH-derived fluorescence is optimal. Due to such an
5   optimization, post-hybridization fluorescence is high enough
to ensure that cells can be measured quantitatively by using
methods such as flow cytometry.

As another embodiment, the invention describes a washing
procedure that allows an effective post-hybridization washing
10   of hybridized cells while maintaining sufficient cell density
for flow cytometric measurement.

The present invention provides for a permeabilization
formula that allows for the first time the performance of in
situ hybridization experiments with all of the bacteria which
15   either contain rigid polysaccharide capsules, extensive
peptidoglycan layers, extraordinary cross-linking moieties in
their peptidoglycan layer and/or that can prevail in the form
of impermeable spores and that breaks down all structural
cell wall components in a controlled fashion. One of the
20   embodiments of the present invention is a permeabilization
formula, another embodiment of the present invention is a
signal amplification methodology which, when used in
combination ensure i) the formation of pores or gaps in the
cell wall of sufficient size as to facilitates the entering
25   of bulky molecules such as horseradish peroxidase enzymes
conjugated to oligonucleotides and ii) that facilitates the
formation through such molecules of sequence-specific
fluorescent precipitates thus allowing the microscopic or
flow cytometric detection of cells stained by fluorescence in
30   situ hybridization.

The invention is further explained in several examples
which can not be seen as limiting the invention.

Examples

Example 1 Cell wall-degrading composition

The consecutive and combined action of several enzymes and
5   chemicals have a degrading activity on the cell wall. A
combination of enzymes results in the systematic - and
thus controllable - degradation of the cell-envelop of
Gram-negative and Gram-positive bacteria. These envelopes
consist of a number of distinct layers, each with their
10   own set of biomolecules. A biochemical background and
information on resistance of these structures to probe
penetration are presented in table 1. A function of the
various components of a permeabilisation or degrading
composition or cells wall degrading reagens is presented
15   in tabel 2.

Example of a possible preparation of a degrading
composition (10.0 ml, sufficient for 50 reactions)

Solution A: 20 ml 48% (w.v) sucrose solution (9.6 g
20   sucrose in 20 ml of Milli-Q water); sterilize by
                     autoclaving;

Solution B: 1 ml 1% (w/v) sodium taurocholate solution
                   (10 mg (Na)taurocholate in 1 ml of sterilized
                   Milli-Q water);

25   Solution C: 1 ml 11.1 % (w/v) calcium chloride solution
                   (11.1 mg $CaCl_2$ in 1 ml of Milli-Q water);
                   sterilize by autoclaving;

Solution D: 100 ml 1M Tris-HCl, pH 7.5 solution (12.1 g
                   Tris(hydroxymethyl)-aminomethane in 90 ml of
30   Milli-Q water, add conc. HCl to pH 7.5, add
                   Milli-Q water to 100 ml); sterilize by
                   autoclaving;

Solution E: 1 ml 20% (w/v) lysozym solution (200 mg
                   lysozym in 1 ml of sterilized Milli-Q water);
35   Solution F: 1 ml 1% (w/v) lipase (10 mg of lipase in 1
            ml of sterilized Milli-Q water);

Solution G: finizym, no preparations needed; purchase
                commercially from Novo Nordisk France;
Solution H: 1 ml 10.000 U/ml mutanolysin (10.000 U
                mutanolysin in 1 ml of sterilized Milli-Q
5   water);

Mix according to the following formula: 2,730 ml solution
A; 0.234 ml solution B; 0.390 ml solution C; 0.195 ml
solution D; 0.117 ml solution E; 0.021 ml solution F;
10   0.042 ml solution G; 0.047 ml solution H. Filter over 0.2
μm membrane filter, transfer to 15 ml polypropylene
bottle, freeze to -80°C and freeze-dry to powder for 48 h.
Before use in permeabilization protocols reconstitute the
lyophilized powder with 9.2 ml of sterilized Milli-Q
15   water. Use 0.2 ml of the reconstituted and cooled (4°C)
formula to treat a cell-pellet of 10^7-10^9 cells. Incubate
the cells for 1-15 min at 4°C in the permeabilization
formula.

20   *Cell permeabilization:*
Example: Lactococcus lactis cells in LD-Bos cheese starter
         culture.
         Clear a LD-Bos cheese starter culture by mixing 1 ml
of culture with 30 ml of 0.2% EDTA. Incubate for 30 min on
25   a roller-bench. Pellet the cells by centrifugation for 10
min at 10.000 rpm. Wash the cell pellet in 15 ml of PBS
(1000 ml of Milli-Q water, 8 g of NaCl, 0.2 g of KCl, 1.81
g $Na_2HPO_4 \cdot 2H_2O$ and 0.24 g of KH_2PO_4, pH 7.4). Pellet the
cells by centrifugation for 10 min at 10.000 rpm.
30   Resuspend the pellet in 4 ml of 4% paraformaldehyde (4 g
of paraformaldehyde; 0.8 g NaCl; 20 mg KCl; 180 mg
$Na_2HPO_4 \cdot 2H_2O$ and 24 mg of KH_2PO_4, pH 7.4). Incubate for 10
min at 4°C. Dilute 400 μl of the fixed cell suspension in
1100 μl of PBS, wash 3 times by successive centrifugation
35   and resuspension of the cells in 1.5 ml of PBS. During
washings, include a short (5 min) incubation in each newly

applied PBS solution. This will allow for the complete
removal of intracellularly accumulated formaldehyde.
Resuspend the washed cell pellet in 200 µl of ice-cold
permeabilization formula and incubate for 15 min at 4°C.

5   Stop the treatment by adding 500 µl of 4% paraformaldehyde
and incubate for 30 min at 4°C. Add 800 µl of PBS and wash
3 times by successive centrifugation and resuspension of
the cells in 1.5 ml of PBS. Resuspend the washed cell
pellet in 500 µl of PBS:ethanol (1:1). The thus treated

10 cells of the LD-Bos cheese starter culture can be used for
in situ hybridization with rRNA, mRNA or DNA-targeted
deoxyribonucleic acid (DNA) oligonucleotide probes,
ribonucleic acid (RNA) oligonucleotide probes, peptide
nucleic acid (PNA) probes that are for their subsequent

15 detection labelled with reporter molecules such as:
- fluorescent labels (e.g., fluorescein, rhodamine,
  methylcoumarine, tertramethylrhodamine,
  aminomethylcoumarin acetic acid, bimane, ethidium,
  europium [iii] cryptate, La Jolla blue,

20 nitrobenzofuran, pyrene butyrate, terbium chelate,
  texas red, or cyanine dyes such as Cy3, Cy5 or
  Cy7);
- enzymatic labels (e.g., oxidases, peroxidases,
  phosphatases, luciferases, dehydrogenases,

25 hexokinases or papain);
- enzyme inhibitors (e.g., phosphonic acid)
- proteinaceous labels (e.g., streptavidin,
  antibodies, Fab fragments or aequorin);
- chemiluminescent labels (e.g., acridinium esters,

30 isoluminol, luminol or ruthenium *tris*[bipyridyl])
- hapten (e.g., biotin, digoxigenin, ethidium,
  glucosyl, sulfone, either
- metal complexes (e.g., 1,4-diaminoethane platinum
  complex)

35 - miscelaneous labels (e.g., small latex particles,
  poly AMP, pyrene)

26

    – phosphors (e.g., Yttriumoxisulfide plus europium or
        zinc silicate plus arsenic and manganese)

    Furthermore, the thus treated cells are permeable to
large biomolecules and the procedure can be used to introduce
5   large (bio)molecules of any kind in the bacterial cell. The
procedure can be shortened by combining separate successive
steps, e.g., (para)formaldehyde fixation can be combined with
ethanol dehydration in one step. Furthermore, the
(bio)chemicals in the protocol can be substituted for any
10   (bio)chemical with comparable activity, e.g., ethanol can be
replaced by methanol or propanol and the enzymes can be
changed for enzymes with comparable biological catalytic
activity provided that the environment of the reaction is
modified according to routine skills to suit the new
15   (bio)chemistry.

Example 2 5'-Horseradish peroxidase labelling of synthetic
oligonucleotides
Step 1. De-protection of commercially derived 5'-thiol
20   *labelled oligonucleotides:*

        Deoxyoligonucleotides (10-30 mers) with a protected
        thiol (SH) group attached to the 5-prime end via a C-6
        spacer arm were commercially derived (Eurogentec,
        Seraing, Belgium). The thiol-groups were de-protected
25   by incubating the oligonucleotide (2 mg/ml) in DP
        buffer (50 mM (Na)phosphate, pH 7.4; 5 mM EDTA; 100 mM
        NaCl; 50 mM hydroxylamine-HCl) for 30 min at 30°C.

    Step 2. Linking of the horseradish peroxidase enzyme to a
30   *sulfo-SMCC linker:*

        Solution 1: 50 mg/ml of sulfo-SMCC (sulfosuccinimidyl
            4-[N-maleimidomethyl]-cyclohexane-1-
            carboxylate; Pierce, Rockford, IL, USA) in DMSO
            (dimethylsulfoxide).
35   Solution 2: 10 mg/ml of enzyme (HRP; Sigma Chemical
            Corp., St. Louis, USA) in PEN buffer, pH 8.0

27

(50 mM (Na)phosphate, pH 8.0; 5 mM EDTA; 100 mM
NaCl).

A volume of 10 µl of freshly prepared solution 1 was
mixed with 200 µl of solution 2 and the mixture was
5   incubated at 30°C for 1 h. After incubation, 790 µl of
PEN buffer, pH 6.7 (50 mM (Na)phosphate, pH 6.7; 5 mM
EDTA; 100 mM NaCl) was added and the SMCC-HRP complex
was purified via ultrafiltration using Centricon 30
(Amicon, Beverly, MA, USA). The complex was washed 3
10   times with PEN buffer, pH 6.7 in Centricon 30
ultrafilters and the supernatant of the final wash was
made up to 200 µl with PEN buffer, pH 6.7.

Step 3. Cross-linking of the HRP-SMCC complex to the de-
15 *protected oligonucleotide-thiol:*

A volume of 67 µl of the deprotected thiol
oligonucleotide (step 1) was mixed with 200 µl of the
purified SMCC-HRP complex (step 2) and the mixture was
incubated at 30°C for 1.5 h. The cross-linked
20   enzyme/oligonucleotide complex was purified via
ultrafiltration using Centricon 30 ultrafilters. The
complex was washed 3 times with PEN buffer, pH 6.7 in
Centricon 30 ultrafilters and the supernatant of the
final wash was made up to 67 µl with PEN buffer, pH
25   6.7. The enzyme-labelled probes were stored at 4°C. For
hybridization, the probe-solution was diluted 10 to 100
times in PEN buffer, pH 6.7.

The (bio)chemicals in the protocol can be substituted for
30 any (bio)chemical with comparable activity, e.g., the
cross-linker sulfo-SMCC can be replaced by any cross-
linker (such as sulfo-GMBS or DTBP) provided that the
environment of the reaction is appropriately modified to
suit the new chemistry. Examples of such linkers are

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succinimidyl-, salicylamido- or hydrazide- derivatives
that contain at least two reactive sites that are
homobifunctional or heterobifunctional reactive towards
(-NH2) amines

5   [e.g., 1,5-Difluoro-2,4-dinitrobenzene (DFDNB),
Dimethyl adipimidate 2HC1 (DMA); or Succinimidyl
4-[N-maleimidomethyl]cyclohexane-1-carboxylate (SMCC)]

(-SH) sulfhydryls
           [e.g., 3-[2-Pyridyldithio]propionyl hydrazide (PDPH);
10   1,4-Di-[3'-(2'-pyridyldithio)-propionamido)]butane
           (DPDPB);   or   Succinimidyl
           4-[N-maleimidomethyl]cyclohexane-1-carboxylate
           (SMCC)];

carbohydrates
15   [e.g., Azidobenzoyl Hydrazide (ABH)]; or
(-COOH) carboxyls
           [e.g., 4-[p-Azidosalicylamido]butylamine (ASBA)   or
           1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide
           Hydrochloride (EDC);
20 or that contain one non-selective photoreactive cross-
linking site and one of the selective reacting sites
mentioned above.

Methodologies for preparing enzyme labelled probes are known
25 in the art. The use of SMCC linkers for this purpose that
activate proteins or enzymes to a thiol function are also
known in the art. (e.g. **Ruth, J.L.** 1994. Oligonucleotide-
Enzyme conjugates. *In:* Protocols for oligonucleotide
conjugates. (Agrawal, S., ed.). *Methods in Molecular Biology*
30 26:167-185; **Alul, R.** 1993. Chemical synthesis of DNA and DNA
Analogs. *In:* DNA probes. Pp. 69-136. (Keller and Manak,
eds.). 2nd ed. Macmillan Publ. Basingstoke, UK.). However,
the methods by which such linkers are used and the methods by
which enzymes are attached to thiolated oligonucleotides is
35 impracticle. Without exception, protocols that are known are

based on chromatographic purification of reaction products
and require the collection of serially eluted fractions from
purification columns. For example, the methodology proposed
by Garman (EP 0422861A) first requires the purification of

5   the maleimido derivatised HRP by a NAP 25 desalting column
and collection of a 1.6 ml fraction (Example 8).
Subsequently, the purification of enzyme-labeled
oligonucleotide (example 9) requires the purification of the
thiolated oligonucleotide over a NAP 5 desalting column, the

10   mixing of the thus thiolated and purified oligonucleotide
with the maleimido-derivatised HRP and finally the
purification of the HRP-labeled oligonucleotide over a Biogel
P-100 column. Such a methodology is laborious, does not
ensure high yields of functional product, and requires

15   experience with column chromatography by the user. Finally,
it is not ruled out that eluted functional products are
missed in the collection process, or that collected fractions
contain no product, or that collected fractions contain
product of insufficient yield.

20

The invention provides a method which employs a
heterobifunctional linker, such as SMCC, in a specific
protocol which is both rapid, yielding a constant product,
not requiring the use of post-purification yield measurements

25   or the identification of the specific product-containing
eluate fractions. The current invention provides a
methodology that is so simple that it can be based on the use
of ultra-filtration devices (filter-columns) in which the
reaction products are retained and of which the filtrate

30   contains the non-desired reactants such as unlabeled probes
or non-reacted linkers. The invention provides as a part of
the invention a protocol for attaching an enzyme via a linker
to an oligonucleotide in a two-step method without the need
of post-synthesis purification and without the need of

35   incorporating post-purification yield controls. This new
method is highly efficient, highly reproducible and excludes

the possibility of losing the product or retrieving non-
functional products (unlabeled probes). As one end-product,
the present method generates enzyme-labeled oligonucleotides
(reaction product A) which can immediately be used in *in situ*

5   hybridization experiments. As non-functional end-products,
the present method may yield un-reacted enzyme (reaction
product B) as well as enzyme with a SMCC moiety attached to
it (reaction product C). Reaction products B and C need not
be purified from reaction product A as both B and C are non-

10   functional in *in situ* hybridization experiments and do not
interfere with the in *in situ* hybridization experiments.
Therefore, the purification of fractions B and C from the
end-product pool would represent unnecessary steps and would
only complicate the procedure. The present invention

15   therefore represents an important improvement of the art. The
proposed method for preparing the HRP-labeled
oligonucleotides is therefore one of the embodiments of the
present invention.

20   Example 3. HRP-Labelling and use of Fluoresceine-Tyramide in
whole cell fluorescence in situ hybridization with
Lactococcus lactis cells.

Fluoresceine-Tyramide (FT) substrate.

25   Tyramine (Sigma) was linked to Fluoresceine-NHS (Boehringer)
by mixing 15 mg of fluoresceine-NHS in 200 μl DMSO with 150
μl tyramine (20 mg/ml H_2O) and using a 4 h incubation at
37°C. The resulting complex was diluted 10 x in DMSO (final
concentration 7 mM).

30
5'-HRP labelling of synthetic oligonucleotides.
Commercially derived deoxyoligonucleotides with 5'-C_6-thiol
(SH) were deprotected with hydroxylamine in PEN buffer (50 mM
(Na)phosphate, pH 7.4; 100 mM NaCl; 5 mM EDTA) for 30 min at

35   30°C. This modified oligonucleotide was subsequently cross-
linked to a horse radish peroxidase (HRP)-Sulfo-SMCC (Pierce)

complex at pH 6.7 for 90 min at 30°C. The HRP-SMCC complex
was formed by incubation of 10 mg/ml HRP with 2.5 mg/ml SMCC
for 1 h at pH 8.0 and 30°C.

5   *Cell wall degradation and storage*
    Cells of *Lactococcus lactis* subsp. *cremoris* were grown in
    overnight culture. Cells from two ml of culture were washed
    in PBS and resuspended in cell wall degrading reagens (50 mM
    Tris-HCl, pH 7.0; 0.03% (w/v) Na-taurocholate; 5 mM $CaCl_2$,
10 20% (w/v) sucrose; 0.5% (w/v) lysozym; 0.003% (w/v)
    pancreatic lipase; 1% v/v finizym. Cells were incubated at
    37°C for 30min, washed in PBS (0.15 M NaCl; 2.5 mM KCl; 10 mM
    Na_2HPO_4, pH 7.4) and fixed for 2 h in 4% (w/v)
    paraformaldehyde. Fixed cells were washed twice in PBS and
15 resuspended in a mixture of PBS and ethanol (96%) (1 : 1).
    Cells were stored at -20 °C for at least 1 h prior to
    hybridisation.

    Hybridisation
20 Hybridisation was carried out by mixing 10 μl of the cell
    suspension in ethanol with 90 μl of hybridisation solution
    (20 mM Tris-HCl, pH 7.2; 0.9 M NaCl; 0.1% SDS; 0.48 M ureum;
    0.25 ng/ml HRP-labelled oligonucleotide probe). Cells were
    hybridized for 16 h at 37 °C.

25
    Probe detection through HRP catalysed reporter deposition.
    After hybridisation, cells were washed 4x with 1/10 strength
    hybridisation buffer. Cells were incubated in fluoresceine-
    tyramide (FT) substrate solution diluted in PER buffer (0.1 M
30 Tris-HCl, pH 8.5; 10 mM imidazol; 0.01% H_2O_2) for 30min at
    RT. After the final washing step in washing solution (100 mM
    Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% v/v Tween 20) cells were
    either used immediately for flow cytometric analysis or were
    filtered onto a 0.2 mm pore-size Isopore™ polycarbonate
35 membrane filter (Millipore Corporation, U.S.A.) for
    epifluorescence microcopic observation. Filters were mounted

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on microscope slides with Vectashield™. Hybridised cells
were viewed under blue light excitation using an Olympus BX40
fluorescence microscope using epi-illumination. Flow
cytometric analysis occurred at 488 nm by using a Becton
5   Dickinson FACScalibur equipped with a 15 mW argon ion laser
and a 10 mW red diode laser. TOTO I (Molecular probes Europe
BV, Leiden, the Netherlands) was used as the counter stain
(figure 1).

10 Example 4. Detection of protease mRNA gene-transcripts in
Lactococcus lactis spp. cremoris
Culture and growth conditions.
Two strains of L. lactis spp. cremoris str. MG1363 (NCDO
736), one which was protease deficient (Prt-) and one in
15 which the defect was cured (Prt+) were grown in M-17 broth
and in skim-milk at 30 °C. Every 30 min, 1 ml samples were
withdrawn aseptically from all four cultures and these
samples were immediately mixed with 100 μl of 37%
paraformaldehyde. Samples were stored at 4°C until analysis.
20

Clarification of milk and preparation of microscope slides
100 μl of the fixed skim-milk samples was diluted into 1 ml
of 10M EDTA (pH 12.0) and incubated at room temperature for 1
h. Samples were centrifuged at low speed (100 x g) and the
25 supernatant was collected. Ten μl of this cell-suspension was
spread onto a microscope slide and dried at 40°C for 30 min.
After incubation with 20 μl of cell-wall degrading reagens
for 15 min at 4°C, cells were hybridized with protease-
specific DNA-probes for 16 h at 45°C.
30

Protease-specific DNA probes.
Three protease specific probes were developed and tested in
this test-system: PRT1245 (5'-gcccctaaagcgactgtacc-3'),
PRT4548 (5'-gataccgccgccgttcaca-3') and PRT6497 (5'-
35 tgtcagacgcatcagcactg-3'). All three probes were 5'end
labelled with thiol (-SH) and were commercially derived

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(Eurogentec, Gent, B). The probes were linked to HRP as
described in example 1.

In situ hybridization

5   A 5'-FITC labelled poly-thymide (poly-T) probe (20-mer)
served as a positive control to demonstrate the presence of
messenger RNA in the cells. A probe complementary to a
universally conserved 16S rRNA bacterial sequence (Eub338)
labelled with HRP served as a positive control, whereas a
10   probe labelled with HRP and complementary to the sequence of
probe Eub 338 (i.e. probe NonEub338) served as a negative
control. Hybridization occurred at 37° C for 16 h. After
hybridization, slides were washed in hybridization buffer for
20 min at 37°C and mounted in Vectashield (Vector Labs, Ca,
15   USA)

Slides were viewed by epifluorescence microscopy on an
Olympus BX-60 microscope equipped with epifluorescence
illumination (HBO-100W Mercury arc lamp and NIBA (bleu)
20   filter-set), a Canon photocamera and a fairchild CCD camera.
Photomicrographs were taken on 400 ASA ECTAChrome diapositive
films by the CCD camera using a 2 sec illuminations.

Example 5. Measurement of activity of single cells via total
25   mRNA quantification by using a poly-T oligonucleotide probe.
The activity of individual cells was measured by whole cell
in situ hybridization of *Escherichia coli* strain JM101 .

Culture and growth conditions.
30   Cells of *Escherichia coli* strain JM101 were grown in TY-
broth at 37 °C. Every 30 min, 1 ml samples were withdrawn
aseptically from the culture and the samples were immediately
mixed with 100 μl of 37% paraformaldehyde. Fixation of the
cells occurred for 30 min at room temperature. Fixed cells
35   were washed twice in PBS and resuspended in a mixture of PBS

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and ethanol (96%) (1 : 1). Cells were stored at -20 °C for at least 1 h prior to hybridisation.

Hybridisation

5   Hybridisation was carried out by mixing 10 µl of the ethanolic fixed cell suspensions with 90 µl of hybridisation solution (20 mM Tris-HCl, pH 7.2; 0.9 M NaCl; 0.1% SDS; 0.25 ng/ml FITC-labelled oligonucleotide probe). Cells were hybridized for 16 h at 45 °C with a FITC-labelled poly-T

10   oligonucleotide probe (20-mer, 5'-FITC), which was commercially derived.

Probe detection.

After hybridisation, cells were washed 4x with

15   hybridisation buffer (hybridization solution without probe). Hybridized cells were either used immediately for flow cytometric analysis and the fluorescence was evaluated qualitatively by epifluorescence microscopy. For epifluorescence microcopy, cells were filtered onto a 0.2

20   mm pore-size Isopore™ polycarbonate membrane filter (Millipore Corporation, U.S.A.) and rinsed 3 x with PBS. Filters were mounted on microscope slides with Vectashield™. Hybridised cells were viewed under blue light excitation using an Olympus BX40 fluorescence

25·   microscope and epi-illumination. Flow cytometric analysis occurred at 488 nm by using a Becton Dickinson FACScalibur equipped with a 15 mW argon ion laser and a 10 mW red diode laser. TOTO I (Molecular probes Europe BV, Leiden, the Netherlands) was used as the counter stain. In all

30   cases 10,000 cells were counted and the fluorescence from FITC was quantified. Figure 3 shows the decay in mRNA-derived fluorescence upon entering of the stationary phase of the culture.

Example 6:   In situ hybridization of *Lactobacillus casei*
(DSM 20011) and *Lactobacillus helveticus* (DSM 20075)

Experimental Procedure

5   Overnight cultures of *Lactobacillus casei* strain DSM 20011
and *Lactobacillus helveticus* strain DSM 20075 in MRS broth at
30°C are washed in PBS and dehydrated for 1 hour at -20C in
PBS/Ethanol (1:1). Cells are pelleted by centrifugation and
the supernatant is discarded. The pellet is resuspended in

10   300 μl of permeabilization mixture and the cells are
incubated at 5°C for various periods of time, up to 180 min.
The following permeabilization mixtures are tested: mixture A
(see permeabilization formula of example 1), and mixture B
(60 μl pronase 100 U/ml, 40 μl proteinase K 20 mg/ml). A

15   volume of 30 microlitres of the permeabilized cell suspension
is mixed with 1 ml of PBS (see example 1) and cells are
pelleted by centrifugation at 14.000 rpm. The pellet is
resuspended in 400 μl of PBS/Ethanol (1:1) and dehydrated at
-20°C for 1 hr. Cells are pelleted by centrifugation at

20   14.000 rpm, the pellet is resuspended in 200 μl of
hybridisation mixture (see example 5) and cells are
hybridized overnight. A volume of 5 microlitres of the cell
suspension is filtered over a 0.2 μm polycarbonate filter,
washed with hybridization buffer and viewed under the

25   microscope using the appropriate filtersets. Fluorescence of
cells is scored visually.

Results
The results of the visual examinations of this study are

30   summarised in Table 4. Clearly, the permeabilization
procedure represents a controlled process by which cells
become more and more permeable to the fluorophore upon
prolonged incubation. Also, at a certain point, lysis starts
to occur resulting in deminishing fluorescence and loss of

35   cells. Depending on the concentration of the reactants in the
mixture (and the incubation temperature, which is not

36

tested), the first signs of permeabilization with a standard
mixture take more than 3 hours to appear in strain DSM 20011,
while strain DSM 20075 can be fully permeabilized in less
than 3 hours. These times are conciderably longer than those

5   needed to permeabilize lactococcus cells (see example 1).
When the concentration of the permeabilization mixture is
increased 5x, permeabilization of strain DSM 20011 is already
complete after 60 min of incubation at 4°C. Mixture B was
derived from a study on the effect of various permeabilizing

10 components described in example 9.

Example 7:   Conventional FISH versus FISH with HRP-labeled
oligonucleotide probes in the flow cytometric measurements of
cells stained by 16S rRNA-FISH.

15

Experimental Procedure
`Conventional' FISH
Faecal bacterial samples are prepared by suspending 0.5 grams
of fresh faecal material in 4.5 ml of PBS by using a dozen

20 sterile glass beads (3 mm diameter) and a vortex. The
suspension is centrifuged at low speed (1,000 rpm) for 1 min
and the supernatant - which contains the cells - is
transferred to a clean microcentrifuge tube. The cell
suspension is then fixed in 4% p-formaldehyde at 4°C for 4

25 hrs. Cells are washed twice in PBS and resuspended in a
mixture of PBS/Ethanol or another organic solvent (1:1) for
dehydration and/or partial degradation. Cells are dehydrated
for at least 1 hr at -20°C. Dehydrated cells are suspended
one in ten in hybridization buffer and hybridized as

30 described above without permeabilization. Hybridization was
performed with probes NonEub, Eub, Bif and Uni labeled with
FITC and NonEub, Eub and Bif labeled with Cy5. Probe
concentrations of 2 ng/μl were used.
Washing was performed both in hybridization buffer from which

35 SDS was ommitted and in ice-cold PBS. In the case of PBS
washings, a volume of 1 ml was added to 500 μl of hybridized

cells. The cell suspension was incubated on ice for 5 min and centrifuged at 14,000 rpm for 5 min. The resulting cell pellet was resuspended in 35 μl of ice-cold PBS and stored on ice. Just prior to flow cytomerty, 5 μl of cell suspension

5   was diluted into 500 μl of ice-cold PBS and cellular fluorescence was acquired by flow cytometry. In the case of hybridization buffer washings, a volume of 1 ml of pre-warmed (45°C) hybridization buffer without SDS was added to the hybridized cells and incubated for 30 min at 45°C. Cells were

10 pelleted by centrifugation at 14,000 rpm for 5 min and resuspended in 1 ml of pre-warmed (45°C) hybridization buffer. The results of the FISH procedure are displayed in table 5 and in figs. 4 and 5.

15 *FISH with HRP probes for signal amplification.*
    A volume of 50 μl of permeabilized LD-BOS cheese starter culture in PBS:Ethanol (1:1) (see example 1), representing a total of 5.10^9 cells per ml, was pelleted by centrifugation. The cell pellet was resuspended in 100 μl of hybridization

20 mixture consisting of 10 μl of non-purified HRP-labeled oligonucleotide (1 in 10 diluted in PEN buffer, see example 2), 8 μl of 6M ureum and 82 μl of hybridization buffer as used above, and incubated for 16 hrs at 37°C. After hybridization, cells were washed for 5 min in 1 ml of

25 hybridization buffer with 0.48 M ureum and pelleted. Washing of the cells subsequently occurred in washing buffers of which the sodium content was gradually decreased (from 0.9 M through 0.75 M and 0.4 M to 0.15 M of NaCl). Finally the pellet was resuspended in FT substrate solution diluted to an

30 effective substrate concentration of 35 μM of FT in PER buffer (see example 3) and incubated for 30 min at room temperature in the dark. Cells were washed twice in washing solution (100 mM Tris-HCl, pH 7,4, 150 mM NaCl, 0.05% v/v Tween 20) and the final cell pellet was resuspended in 50 μl

35 of ice-cold PBS and stored on ice. A volume of 5 μl of this suspension was added to 500 μl of ice-cold PBS (pH 7,4) and

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immediately used for flow cytometric measurement. The results
of the tests with HRP labeled probes are summarized in table
6 and fig. 6.

5   **Results**

   'Conventional' FISH
   The results of the 'conventional' FISH procedure are
   displayed in table 5 and figs. 4 and 5. As can be concluded
10   from the signals derived with the FITC-labeled probe Eub -
   which stains virtually all bacteria - a pH of around 10.4 in
   the measuring buffer yields optimal fluorescence intensities.
   When hybridising with the *Bifidobacterium*-specific Bif probe,
   distinct subpopulation of cells with above-background
15   fluorescence (*Bifidobacterium* spp.) could only be established
   for FITC labeled probes at pH values 9.5 and 10.4 (see fig.
   4A), or when using Cy5 labeled probes (see fig. 4B). Although
   the staining of bifidobacteria by using the Cy5-labeled Bif
   probe resulted in a slightly better separation between
20   positive (Bif labeled) and negative (non-stained) cells (see
   fig. 4) fluorescence intensities were overall comparable.
   As another optimisation of 'conventional' FISH for flow
   cytometric application Cy5 labeled oligonucleotide probes
   were combined with YOPRO-1 and Cyto-9 (both derived from
25   Molecular Probes Inc.) as general nucleic acid counter stains
   (see fig. 5). In this way, nucleic acid-containing particles
   (bacteria) could be distinguished from DNA-void bodies
   (detritus) prior to measuring their fluorescence. Optimal
   staining of the cellular nucleic acids was achieved when
30   using Cyto-9 as a nucleic acid stain. However, although Cyto-
   9 (panel C) resulted in the highest fluorescence intensities
   (compare upper panels), YOPRO-1 at 10x diluted commercial
   concentration (panel A) resulted in better separation
   between positive and negative cells (lower panels) than
35   YOPRO-1 at 40x diluted commercial concentration (panel B,
   lower panel) or Cyto9 (panel C, lower panel) (see also fig. 4

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panel B, where excellent separation of the bifidobacteria is achieved in combination with YOPRO-1 at 10x diluted commercial concentration).

As a result of these tests, we concluded that procedures for
5   `conventional' FISH were optimised and true comparison with HRP protocols was thereafter allowed.

FISH with HRP probes for signal amplification.

The results of the tests with HRP labeled probes are
10 summarised in table 6 and fig. 6. The enormous increase in the intensity of fluoresceine-derived fluorescence as a result of the HRP labeling and fluoresceine-tyramide precipitation is striking. Compared to `conventional' labeling procedures, where optimisation by pH adjustment can
15 result in a 4x increase in fluorescence (see table 5), HRP labeling results in a 30x increase in fluorescence as compared to conventional procedures (see table 6). It should be stated that the pH of the measuring buffer in these HRP experiments was 7.4 and therefor not optimal and that a
20 further increase in signal is possible when optimal conditions - such as higher measuring pH - are used (up to 3 times). The high background fluorescence of cells as notable in test with the HRP labeled NonEub probe was due to non-specific binding and could be reduced by using blocking
25 reagents known to the art, such as Denhardts solution. Figs. 6 demonstrates the shift in fluorescence upon labeling with HRP probes in FISH in graphical display. The intensity of HRP labeling is comparable to the staining with the nucleic acid stain YOPRO-1 at a dilution of 40x (see fig 5, panel B).
30 These types of intensities are required for flow cytometric detection of FISH in bacterial cells, especially in cells that exist in a low level metabolic state, i.e. in which conventional FISH results in fluorescence levels too low for convenient detection such as soil bacteria or bacteria in
35 natural water bodies.

40

Example 8:   Detection of nisin mRNA gene-transcripts in
Lactococcus lactis.

Experimental Procedure

5   In this experiment the procedure used in example 4 is used to
detect messenger RNA's in whole cells of *Lactococcus lactis*
for the antimicrobial peptide nisin. Hereto, cells of strain
161-5 of nisin producing *Lactococcus lactis* were subjected to
nisin-inducing growth conditions. Samples were withdrawn
10   every hour for 8 hrs and the cells were treated and
hybridized with a HRP-labeled oligonucleotide specific for
nisin-mRNA as described in example 4. A non-producing strain
(strain 1614) served as a negative control in this
experiment. Fluorescence was monitored visually and
15   photographs were taken for later verification.

Results

The results of these tests are summarised in fig. 7. Cells of
strain 1614 (fig. 7 panel A) did not show any change in the
20   intensity of fluorescence during the course of the
experiment. However, cells of strain 161-5 (fig. 7 panel B)
displayed high fluorescence intensities during the first 2
hours of growth under inducing conditions and a diminishing
degree of fluorescence in the following 2 hrs. After 4 hours,
25   fluorescence returned to background levels.
This experiment shows that detection of mRNA gene-transcripts
can readily be undertaken provided that appropriate
permeabilization procedures are used.

30   Example 9:   Detection of *Lactobacillus helveticus* by 16S
rRNA-targeted fluorescence *in situ* hybridization.

Experimental Procedure

Overnight cultures of *Lactobacillus helveticus* strain DSM
35   20075 in MRS broth at 30°C are washed. Cells are pelleted by
centrifugation and the supernatant is discarded. The pellet

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is resuspended in 300 µl of permeabilizing mixture and the
cells are incubated for 15 minutes at room temperature. The
various permeabilization mixtures used are summarised in
table 8. Following the permeabilization cells are fixed in
5   3.7% formaldehyde at 4°C for 30 min. The cells are washed
twice in PBS, spotted to convenient densities on multi-wells
microscope slides and dried at 45°C 15 min. The cells are
dehydrated in a graded ethanol series (50%, 70% and 100% of
ethanol) by submersing the entire slides in the various
10   ethanol solutions for 2 min. The slides are dried to air and
a volume of 50 µl of hybridization mixture with FITC labeled
probes is applied to the slides. The entire slide is covered
by a large cover glass, and incubated in a hybridization
chamber for a period of 4 hrs. Upon hybridization, slides are
15   washed in hybridization buffer at 45°C for 5 min, dried in
air and mounted for microscopic examination.

Results

The results of the permeabilization experiments with
20   *Lactobacillus helveticus* strain DSM 20075 are summarised in
table 9 and fig. 8. Certain mixtures (mixtures 1 and 2)
result in the immediate lysis of the cells. Such mixtures are
not directly suited for controlled lysis needed to
permeabilize the cells. However, lower concentrations of the
25   compounds used in these mixtures may still yield useful
results in permeabilization procedures. Clearly, for
Lactobacillus helveticus strain DSM 20075, a mixture
consisting of a combination of proteinases (mixtures 12, 16,
17 and 18) results in excellent permeabilization of
30   *Lactobacillus helveticus* cells. Such a mixture was also used
in example 6. In fig. 8, pictures of *Lactobacillus helveticus*
strain DSM 20075 hybridized with probe Eub without
permeabilization (panel A) and permeabilized with mixture 16,
17 or 18 consisting of a combination of proteinase K and
35   protease type XXI (panel B) are presented.

Table 1. Bacterial cell wall components, their biochemical details and the resistance of these structures to probe penetration.

Cellular layer	Dominant macromolecules	Chemistry	Penetration resistance	Degrading component
Lipopolysaccharide	LPS (polysaccharides and lipids)	sugar / lipid (fatty acid and phosphate)	surface coverage	finizym / lipase+ taurocholate
phospholipid		lipid	surface polarity	lipase+ taurocholate
peptidoglycan (mucopeptide)	N-acetylglucosamines (NAG)	sugar / peptide	structural barrier	lysozyme
	N-acetylmuramic acids (NAM)	sugar / peptide	structural barrier	finizym, Ca^{2+} /lipase+ taurocholate
	tetrapeptides	peptide		
	(lipo)teichoic acids and teichuronic acids	sugar / lipid	structural barrier	autolysins
cytoplasmic membrane	phosphatidylethanolamines and phosphatidylglycerol	lipid	surface polarity	lipase+ taurocholate

Table 2. Bacterial cell wall components, biochemical details and resistance to probe penetration.

Component	Function
saccharose (sucrose) or lysine betaine	Osmoticum, prevents cells from bursting
sodium taurocholate	detergent, increases surface area of lipids
calcium chloride	Decreases cell wall rigidity by competing with bivalent cations that keep in check the electronegative repulsion from teichoic and teichuronic acids
Tris-HCL	buffer
lysozyme	glycosidase that splits disaccharide bond between N-acetylglucosamines (NAG) and N-acetylmuramic acids (NAM) thereby degrading peptidoglycan
lipase	degrades lipids by hydrolysing ester-bond between glycerol moiety and fatty acid tails
finizym	beta-glucanase that hydrolyses 1,4- and 1,3-beta-glucan bonds thus producing oligosaccharides and disaccharides
mutanolysin	degrades *Streptococcus faecalis* cell walls

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Table 3.

Probes for DNA and mRNA of proteinase of *Lactococcus*
lactis subspecies *lactis*

5   PRT1245:   5'-GCCCCTAAAGCGACTGTACC-3'

PRT4548:   5'-GATACCGCCGCCGTTCACA-3'

PRT6497:   5'-TGTCAGACGCATCAGCACTG-3'

Probes for *Lactobacillus casei* 16S rRNA

10   LBC186   5'-TTCAGCCAAGAACCATGCG-3'

LBC194   5'-CTTACGCCATCTTTCAGCCA-3'

LBC446   5'-ACGCCGACAACAGTTACTC-3'

LBC453   5'-CTCTGCCGACCATTCTTCT-3'

LBC1000   5'-CCTGATCTCTCAGGTGATC-3'

15   LBC1021   5'-GCCCCCGAAGGGGAAAC-3'

LBC1008   5'-GGAAACCTGATCTCTCAGG-3'

LBC1448   5'-GGCTCGCTCCCTAAAAGG-3'

Probe for *Lactococcus lactis* subspecies *cremoris* 23S rRNA

20   LLC1199   5'-CTCCTACCATTGATAAAATATCAAT-3'

LLC1202   5'-CTACCATTGATAAAATATCA-3'

Probe for *Lactococcus lactis* subspecies *lactis* 23S rRNA

25   LLL1199   5'-CTCCTACCATTTAATTAAATTAAAT-3'

LLL1202   5'-CTACCATTTAATTAAATTAA-3'

Table 3, continued

Probes for *Mycobacterium avium* subspecies *paratuberculosis*
23S rRNA
5   MPA748   5'-CGCCACTACACCCCAAAAG-3'
    MPA1320   5'-GATCACCCGGAGCTTCG-3'
    MPA1705   5'-GTGACGGACGGATTTGCC-3'
    MPA1712   5'-CAGGATTAGTGACGGACGG-3'
    MPA1796   5'-GGCAGTGTATGTGCTCGC-3'
10 MPA2760   5'-CGAACAGCCCAACCCTTG-3'
    MPA3029   5'-TGATCTGCGGGGGGCCT-3'

Probe for *Mycobacterium avium* subspecies *paratuberculosis*
ITS sequence (Internally Transcribed Spacer between 16S
15 and 23S rRNA codons)
    MPAITS   5'-AACGCCGGCCACGGAGA-3'

Probes for "Dermatitis Digitalis" related *Treponema*
species 16S rRNA
20 TDD141   5'-CGGATACCCATCGTYGCC-3'
    TDD154   5'-TCTAGSAGCTATCCCCATC-3'
    TDD275   5'-ATTCGGTATTACCTSCTATTTC-3'

Probes for *Clostridium difficile* 16S rRNA
25 CDI174   5'-CTCTCAAATATATTATCCCGT-3'
    CDI190   5'-CACCTTTGATATTCAAGAGA-3'
    CDI198   5'-ATCCTGTACTGGCTCACC-3'
    CDI825   5'-GTAACCCCCGAACACCTAGT-3'
    CDI845   5'-ACGTGCGGCACCGAGGGGGG-3'

Table 4. Effect of permeabilization mixture on hybridization efficiencies for the detection of *Lactobacillus* species (results for example 6).

Permeabilization time	Probe	Mixture A			Mixture A (5X)	Mixture B		
		Feacal sample	Lactobacillus helveticus DSM 20075	Lactobacillus casei DSM 20011	Lactobacillus casei DSM 20011	Feacal sample	Lactobacillus helveticus DSM 20075	Lactobacillus casei DSM 20011
15 min	NonEub	- 100%	- 100%	- 100%		- 100%	- 100%	- 100%
	Eub	+ >95%	+ >95%	+ <1%		++ >95%	++ >95%	+ 10%
	Lab	+ <5%	+ 15%	+ <1%		+ 30%	++ 50%	+ 10%
30 min	NonEub	- 100%	- 100%	- 100%		- 100%	- 100%	- 100%
	Eub	++ 10%	++ 80%	++ <1%		++ 40%	++ 70%	+ 5%
	Lab	- 100%	++ 50%	++ <1%		+/- 5%	++ 50%	+ 5%
60 min	NonEub	- 100%	- 100%	- 100%	- 100%	- 100%	- 100%	- 100%
	Eub	++ 75%	++ 70%	+ 2%	++ 98%	++ 85%	++ 70%	+ 10%
	Lab	- 100%	++ 50%	+ 2%	++ 95%	+ 5%	++ 50%	+ 10%
90 min	NonEub	- 100%	- 100%	- 100%		- 100%	- 100%	- 100%
	Eub	++ 50%	++ 85%	+ 2%		++ 70%	++ 90%	++ 7%
	Lab	- 100%	++ 55%	+ 2%		+/- n.d.	++ 55%	+ 7%
120 min	NonEub	- 100%	- 100%	- 100%		- 100%	- 100%	- 100%
	Eub	- 100%	+ 70%	+ 4%		++ 95%	++ >95%	++ 8%
	Lab	- 100%	+ 65%	+ n.d.		- 100%	++ 85%	+ 10-12%
150 min	NonEub	- 100%	- 100%	- 100%		- 100%	- 100%	- 100%
	Eub	++ 10%	+ 60%	+ 5%		++ 80%	++ 90%	++ 7%
	Lab	- 100%	+ 50%	+ 5%		+ 100%	++ 90%	+ 7%
180 min	NonEub	- 100%	- 100%	- 100%		- 100%	- 100%	- 100%
	Eub	++ 25%	+ 80%	+ n.d.		++ 60%	++ 95%	+ 10%
	Lab	- 100%	+ 50%	+ <20%		+/- n.d.	++ 95%	+ 20%

Table 5. Effect of label and pH on the fluorescence intensity by 'conventional' FISH of bacteria in human intestinal microflora as determined by flow cytometry.

| | FITC | | | | | | Cy5 | |
| | pH 7.4 | | pH 9.4 | | pH 10.4 | | pH 7.4 | |
Probe	Mean	Median	Mean	Median	Mean	Median	Mean	Median
Non-Eub	3,92	1,83	5,88	2,79	4,90	2,23	53,21	12,19
Bif	n.d.	n.d.	12,10	4,74	12,05	4,87	35,93*	7,57
Eub	12,90	7,37	43,96	21,87	48,80	24,14	62,73	33,68
Uni	9,16	4,83	33,11	16,25	31,19	15,40	n.d.	n.d.
Uni + Eub	19,76	10,00	67,14	31,91	80,82	38,54	n.d.	n.d.

* Percentage of cells with above-background fluorescence (*Bifidobacteria*): 7,20%

Table 6. Effect of HRP labelling on the fluorescence intensity of *FISH* in LD-BOS cheese starter culture as determined by flow cytometry.

Probe	FITC			HRP-FITC	
	Mean	Median		Mean	Median
Non-Eub	4,44	2,18		80,87	39,79
Eub	15,80	8,45		468,80	135,77
LLC	17,63	9,38		549,48	198,90

n.b. The pH of the measuring buffer was 7.4 in these experiments.

Table 7. 16S rRNA Probes used in this study

Probe Name	Specificity	Sequence (5'-3')	Labels
Non-Eub	negative control probe	ACTCCTACGGGAGGCAGC	FITC, Cy5, HRP
Eub	Virtually all Bacteria	GCTGCCTCCCGTAGGAGT	FITC, Cy5, HRP
Bif	Genus *Bifidobacterium*	CATCCGGCATTACCACCC	FITC, Cy5
LLC	*Lactococcus lactis* subsp. *cremoris*	TGCAAGCACCAATCTTCATC	FITC, Cy5, HRP
Uni	Universal (Eukaryae, Archaea and Bacteria)	G(AT)ATTACCGCGGC(GT)GCTG	FITC, Cy5
Lab	Genus *Lactobacillus*	GGTATTAGCA(TC)CTGTTTCCA	FITC

Table 8. Composition of Permeabilization Mixtures used in example 9.

'Mixture' 0	- milli-q 300 µl	Mixture 17	- proteinase K 40 µl (of a 20 ng/µl solution) - pronase 60 µl (of a 100 U/ml solution) - milli-q 200 µl
Mixture 1	- polymyxine 80 µl (of a 0,00075 g/ml solution) - streptomycine 40 µl (of a 0,003 g/ml solution) - lipase 160 µl (of a 1 mg/ml) - CaCl₂ 200 µl (of a 22,2%) - Lysozym 80 µl (of a 200 mg/ml solution) - mutanolysine 40 µl (of a 10.000 U/ml solution) - finizym 40 µl - milli-q 560 µl	Mixture 18	- proteinase K 60 µl (of a 20 ng/µl solution) - pronase 90 µl (of a 100 U/ml solution) - milli-q 150 µl
		Mixture 19	*Permeabilisation formula (see example 1):*
Mixture 2	- polymyxine 80 µl (of a 0,00075 g/ml solution) - streptomycine 40 µl (of a 0,003 g/ml solution) - lipase 160 µl (of a 1 mg/ml solution) - CaCl₂ 200 µl (of a 22,2% solution) - Lysozym 80 µl (of a 200 mg/ml solution) - mutanolysine 40 µl (of a 10.000 U/ml solution) - milli-q 600 µl		
Mixture 3	- lysozym 20 µl (of a 200 mg/ml solution) - mutanolysine 10µl (of a 10.000 U/ml solution) - finizym 10 µl - milli-q 260 µl		
Mixture 4	- polymyxine B 20 µl (of a 0,00075 g/ml solution) - streptomycine 10µl (of a 0,003 g/ml solution) - lipase 40 µl (of a 1 mg/ml solution) - CaCl₂ 50 µl (of a 22,2 % solution) - finizym 10 µl - milli-q 170 µl		
Mixture 5	- tolueen 3 µl - milli-q 297 µl		
Mixture 6	- tolueen 30 µl - milli-q 270 µl		
Mixture 7	- DIDS 150 µl (of a 2mg/ml solution) - milli-q 150 µl		
Mixture 8	- DTT 150 µl (of a 1M solution) - milli-q 150 µl		
Mixture 9	- DIDS 150 µl (of a 2 mg/ml solution) - DTT 150 µl (of a 1M solution) - Tolueen 30µl		
Mixture 10	- LiCl 300 µl (of a 5M solution)		
Mixture 11	- LiCl 270 µl (of a 5M solution) - lysozyme 20 µl (of a 200 mg/ml solution) - mutanolysine 10 µl (of a 10.000 U/ml solution)		
Mixture 12	- proteinase K 20 µl (of a 20 ng/ml) - pronase (protease type XXI) 30 µl (of a 100 U/ml solution) - milli-q 250 µl		
Mixture 13	- LiCl 300 µl (of a 10M solution)		
Mixture 14	- LiCl 300 µl (of a 15M solution)		
Mixture 15	- LiCl 270 µl (of a 10M solution) - lysozyme 20 µl (of a 200 mg/ml solution) - mutanolysine 10 µl (of a 10.000 U/ml solution)		
Mixture 16	- proteinase K 20 µl (of a 20 ng/µl solution) - pronase 30 µl (of a 100 U/ml solution) - milli-q 250 µl		

Table 9. Effect of different mixtures on the permeabilization
of Lactobacillus helveticus strain DSM 20075 for FISH.

Mixture	Probe		
	NonEub	Eub	Lab
0	-	-	-
1	lysis	lysis	lysis
2	lysis	lysis	lysis
3	-	-	n.d.
4	-	-	n.d.
5	-	-	n.d.
6	-	-	n.d.
7	-	-	n.d.
8	-	-	n.d.
9	-	-	n.d.
10	-	+	+/-
11	-	+	n.d.
12	-	++	n.d.
13	-	+	+/-
14	-	+	+/-
15	-	+	+/-
16	-	+	+/-
17	-	++	+
18	-	+++	++
19	-	+	+/-

-   : no fluorescence   n.d., not determined

-/+ : weakly fluorescent

+   : properly fluorescent

++ : very good fluorescence

+++: bright fluorescence

Figures

Figure 1. Illumination series demonstrating the enormous
(80x) increase in fluorescence emission from HRP-labelled
5   probe (photograph D: 0.5 sec illumination) compared to
conventional FITC-labelled oligonucleotide probes
(photographs A, B and C: 0.5, 4 and 10 sec illumination,
respectively). Hybridizations were performed on cheese
starter-culture LD-Bos. The HRP-labelled probe used was
10 targeted to the 23S rRNA of *Lactococcus lactis* subspecies
cremoris cells (over 50%). The FITC-labelled probe was
targeted to the 16S rRNA of all bacterial cells present in
the sample. The protocol for in situ hybridization with HRP-
labelled probes of example 1 was applied. The average
15 fluorescence of cells in photograph D was 181, that of
photograph C was 53 (in AU) as determined by image cytometry.

Figure 2. CCD-images of the *prt+* strain of *L. Lactis* spp.
cremoris str. MG1363 (NCDO 736) hybridized with a mixture of
20 the three PRT probes as described in example 2. Comparison of
phase contrast (A) and fluoresceine fluorescence (B) images
show that the protease transcript is not present in equal
amounts in individual cells.

25 Figure 3. Measurement of mRNA via an FITC-labelled poly-T
oligonucleotide probe in individual Escherichia coli cells
by flow cytometry.

Figure 4
30 Flow cytometer cytograms representing the measurements of
human intestinal (faecal) flora with *Bifidobacterium* genus-
specific 16S rRNA targeted *in situ* hybridization probes
labeled at the 5'end with FITC (panel A) and Cy5 (panel B).
Fluorescence intensities (y-axis) are plotted versus Forward
35 Scattering Signal (x-axis). With both labels, a distinct
group of cells with higher fluorescence than the body of the

population can be distinguished. Pthe pH of the measuring
medium was 10.4 for FITC and 7.5 for Cy5 with TOPRO-3 and
YOPRO-1 as the DNA counter stains for FITC and Cy5,
respectively.

5

Figure 5
The effect of DNA counter stains on the fluorescence
intensity derived from FISH. Flow cytometer histograms
representing the measurements of human intestinal (faecal)
10   flora with *Bifidobacterium* genus-specific 16S rRNA targeted
in situ hybridization probes labeled at the 5'end with Cy5.
The nucleic acid-counterstains YOPRO-1 and Cyto9 were used.
Panel A, top: The YOPRO-1 fluorescence (x-axis) versus the
cell counts. YOPRO-1 was applied as a 10x diluted commercial
15   preparation. Panel A, bottom; The Cy5 fluorescence derived
from these cells (x-axis) versus the cell counts. Panel B,
top: The YOPRO-1 fluorescence (x-axis) versus the cell
counts. YOPRO-1 was applied as a 40x diluted commercial
preparation. Panel B, bottom; The Cy5 fluorescence derived
20   from these cells (x-axis) versus the cell counts. Panel C,
top: The Cyto-9 fluorescence (x-axis) versus the cell counts.
Panel C, bottom; The Cy5 fluorescence derived from these
cells (x-axis) versus the cell counts.

25   Figure 6
The effect of HRP labeling on the fluorescence intensity
derived from FISH. Flow cytometer histograms representing the
measurements of LD-BOS cheese starter culture with a
Lactococcus lactic subspecies *cremoris* -specific 23S rRNA
30   targeted *in situ* hybridization probe (LLC) labeled at the
5'end with HRP (panel B). No nucleic acid-counterstains were
used. Panel A, top: The FITC fluorescence derived from HRP-
labeled probe NonEub used in combination with FT-substrate
(x-axis) versus the cell counts. Panel A, bottom; The far-red
35   fluorescence derived from these cells (x-axis) versus the
cell counts. Panel B, top: The FITC fluorescence derived from

HRP-labeled probe LLC used in combination with FT-substrate
(x-axis) versus the cell counts. Panel B, bottom; The far-red
fluorescence derived from these cells (x-axis) versus the
cell counts.

5

Figure 7.
Nisin mRNA detection in *Lactococcus lactis*. Panel A: Non
nisin-producing strain 1614 after 3 hrs of growth under
nisin-inducing conditions. Panel B: Nisin-producing strain

10   161-5 at various times during 6 hrs of growth under nisin-
inducing conditions. See text for details.

Figure 8
The *in situ* hybridization of *Lactobacillus helveticus* strain

15   DSM 20075 with FITC-labeled probes without (panel A) and with
permeabilization (panel B). Left-hand side images represent
FITC fluorescence, right hand side images are corresponding
phase-contrast pictures. Upon effective permeabilization, the
cells become almost transparent.

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CLAIMS

1. A method for *in situ* staining of micro-organisms comprising: a) mixing a material containing at least one (fixed or non-fixed) micro-organism with a composition which can (partly) degrade a cell wall or cell membrane of

5   a micro-organism thereby allowing for penetration through said wall and/or membrane of a (labelled) probe into said micro-organism, b) optionally fixing said micro-organism with a fixative to further retain its individual corpuscular character, c) reacting said probe with an

10   antigen or nucleic acid molecule present in said micro-organism, and d) detecting the presence of said probe in said micro-organism.

2. A method according to claim 1 wherein said micro-organism belongs to any of the group of viruses, bacteria,

15   yeast, fungi and plant cells.

3. A method according to claim 1 or 2 wherein said composition at least comprises one detergent, such as sodium taurocholate and at least one enzyme, such as a lipase.

20   4.   A method according to claim 1 or 2 wherein said composition at least comprises a proteinase.

5.   A method according to claim 4 wherein said proteinase is proteinase K and/or protease type XXI.

6.   A method according to claims 3 to 5 wherein said

25   composition additionally comprises a carbohydrate degrading enzyme such as lysozyme, finizym, or mutanolysin.

7.   A method according to anyone of claims 3 to 6, wherein said composition additionally comprises at least

30   one osmoticum, such as saccharose, sucrose, or lysine betaine, and/or at least one cation, such as Ca^{++} or Li^+.

8.   A method according to any of claims 1 to 7, wherein said fixative is selected from any of the group composed of (para)formaldehyde, glutaraldehyde, ethanol, methanol.

9.   A method according to any of claims 1 to 8 wherein
said probe is a nucleic acid probe.

10.   A method according to claim 9 wherein said probe is
directed against genomic or ribosomal DNA or against

5   messenger or ribosomal RNA.

11.   A method according to any of claims 9 or 10 wherein
said probe is labelled to a reporter molecule.

12.   A method according to claim 11 wherein said reporter
molecule is selected from any of the group of

10   fluorochromes or enzymes.

13.   A method according to claim 12 wherein the reporter
molecule is horseradish-peroxidase specifically linked,
preferably with a sulfo-SMCC linker, to a nucleic acid
probe.

15   14.   A method acccording to any of claims 1 to 13 wherein
the presence of said probe or its reaction products is
detected by microscope, image cytometry, fluorometry or
flow cytometry.

15.   A method according to claim 14 wherein the presence

20   of said probe is detected by detecting fluorescence.

16.   A method according to claim 15 wherein the
fluorescence of tyramine-fluorochromes is detected.

17.   A method according to any of claims 13 to 15 wherein
the micro-organism is not immobilised to a slide or

25   filter.

18.   A composition comprising a cell-wall degrading
reagens for use in in situ staining which comprises at
least one detergent, such as sodium taurocholate and at
least one enzyme, such as a lipase, or at least a

30   proteinase.

19.   A composition according to claim 18 which further
comprises a carbohydrate degrading enzyme such as
lysozyme, finizym, or mutanolysi

20.   A composition according to claim 18, or 19 which

35   further comprises at least one osmoticum, such as
saccharose, sucrose, or lysine betaine, to regulate

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osmotic pressure and/or at least one cation, such as Ca^{++} or Li^{+}.

21.　A nucleic acid probe derived from a thiolated probe linked to horseradish-peroxidase with a sulfo-SMCC linker.

5　22.　A method for linking a nucleic acid probe to horse-radish peroxidase comprising mixing a thiol oligonucleotide with a SMCC-HRP complex, incubating said mixture and purifying it by ultrafiltration.

23.　A diagnostic test kit for use with *in situ* staining

10　which at least comprises a cell wall-degrading composition according to any of claims 18 to 20 and/or a nucleic acid probe according to claim 21.

24.　An enzyme-linked assay or diagnostic kit which comprises a nucleic acid probe according to claim 21.
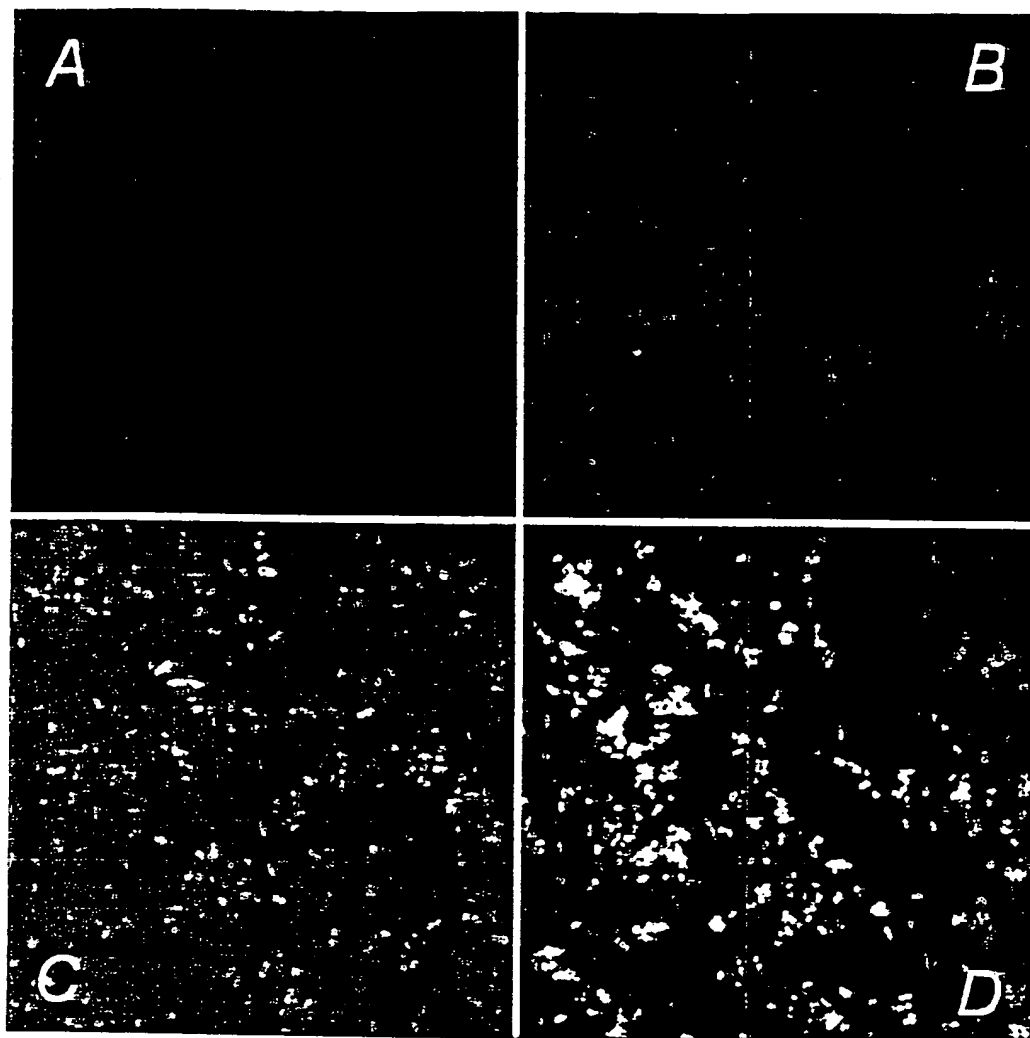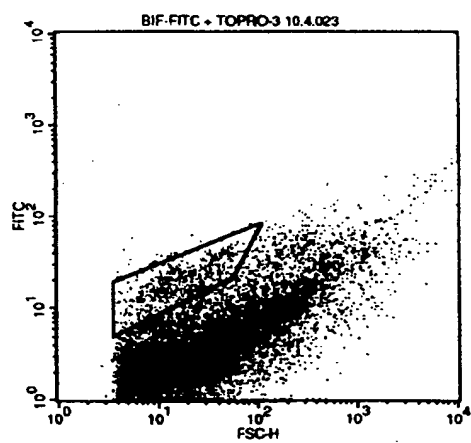
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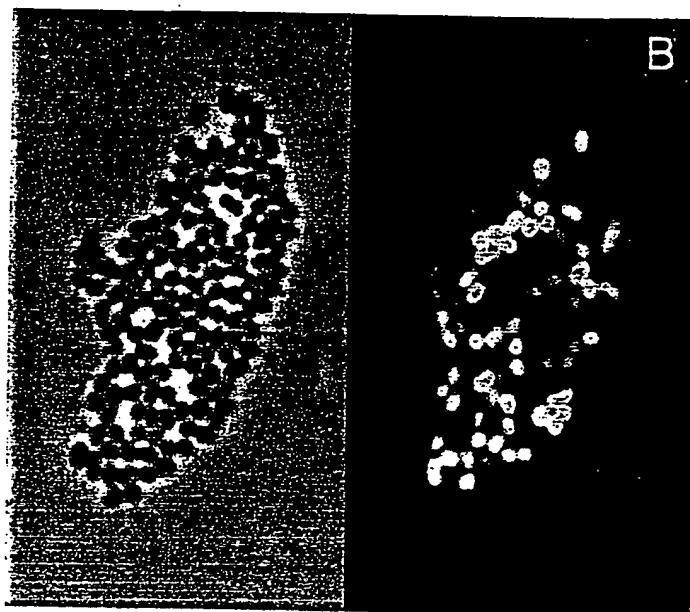
Figure 2
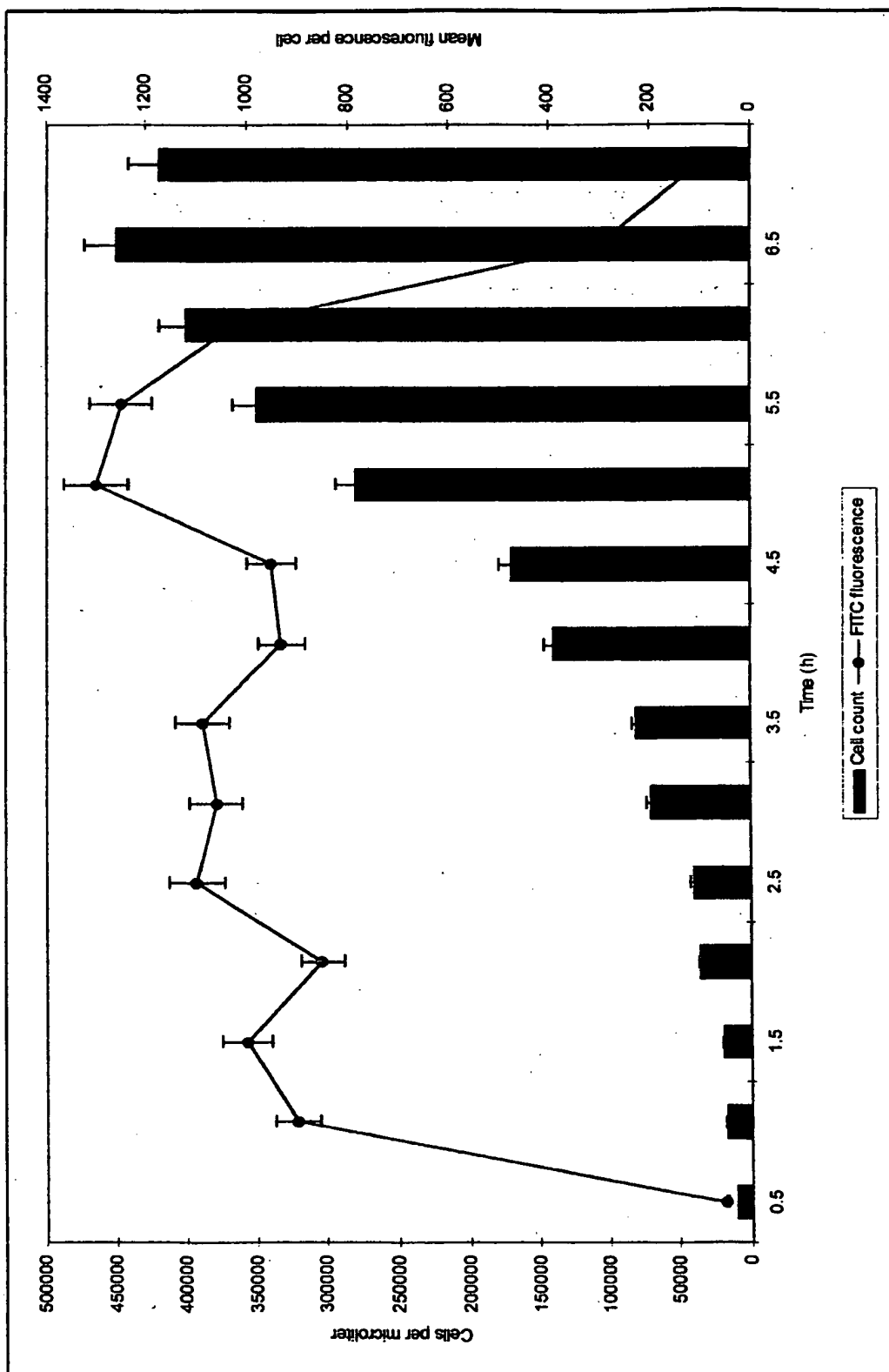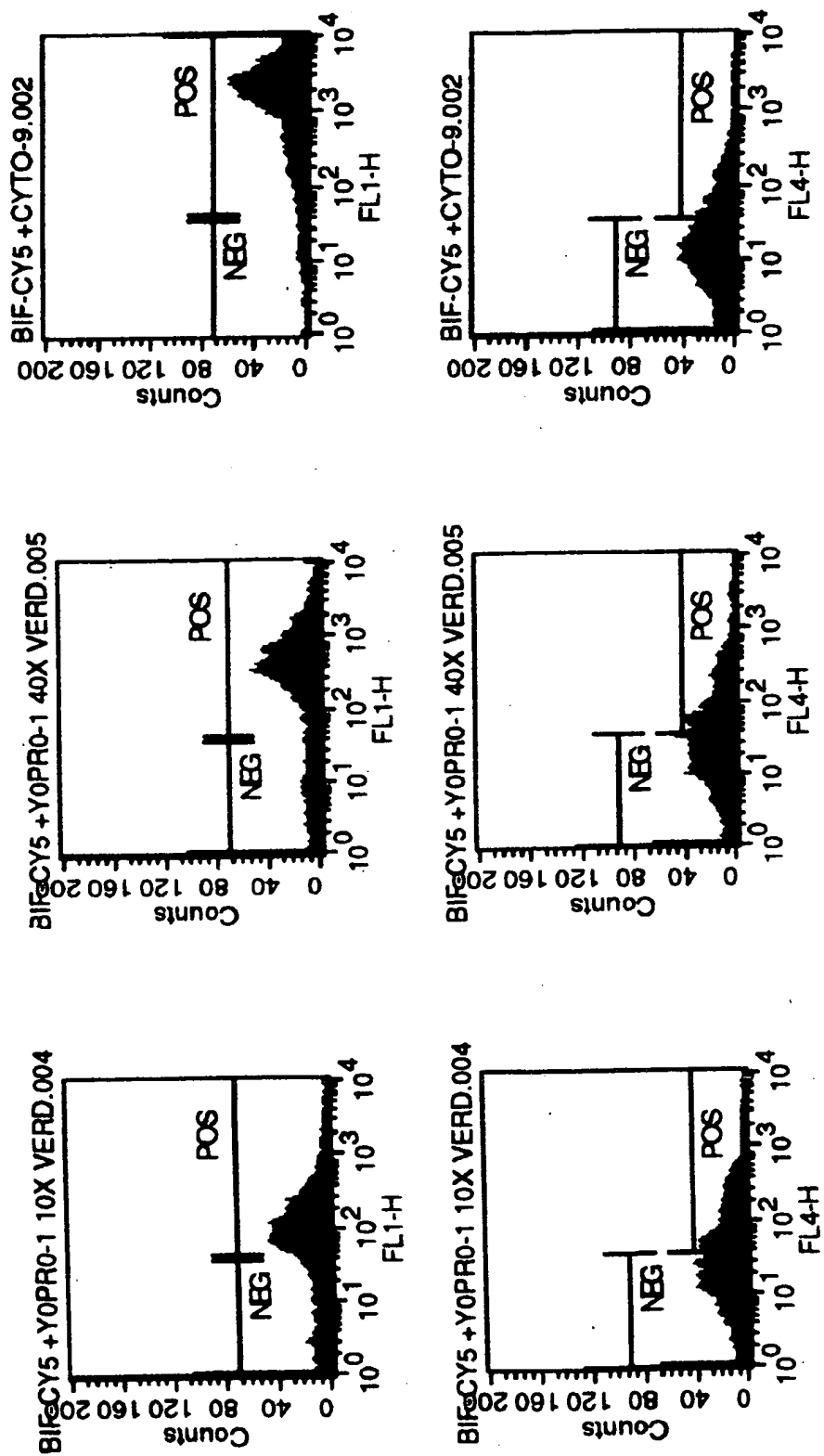
Figure 3

Fig. 4

A   B
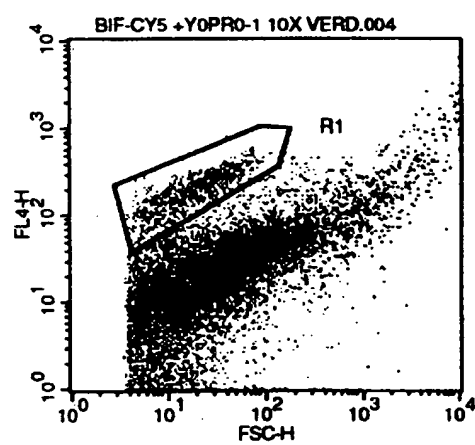
Fig. 5

Fig. 6
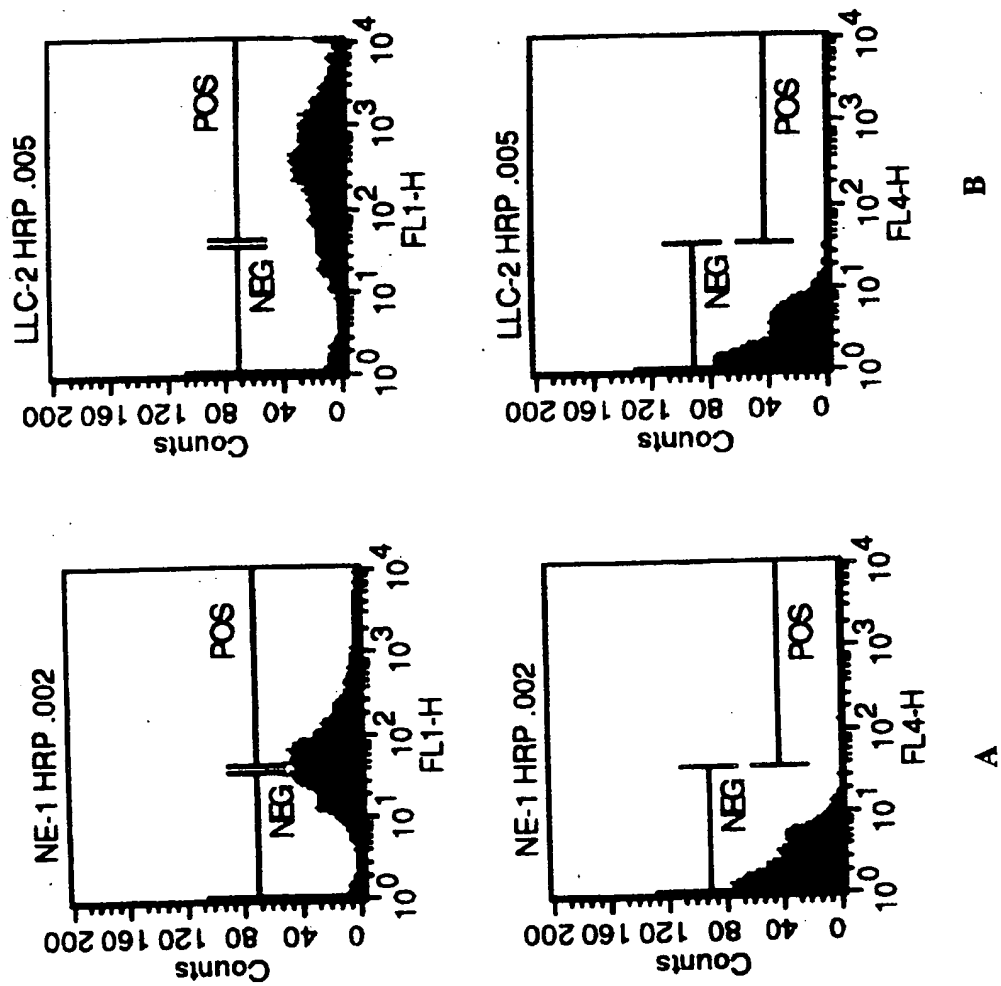
Fig. 7
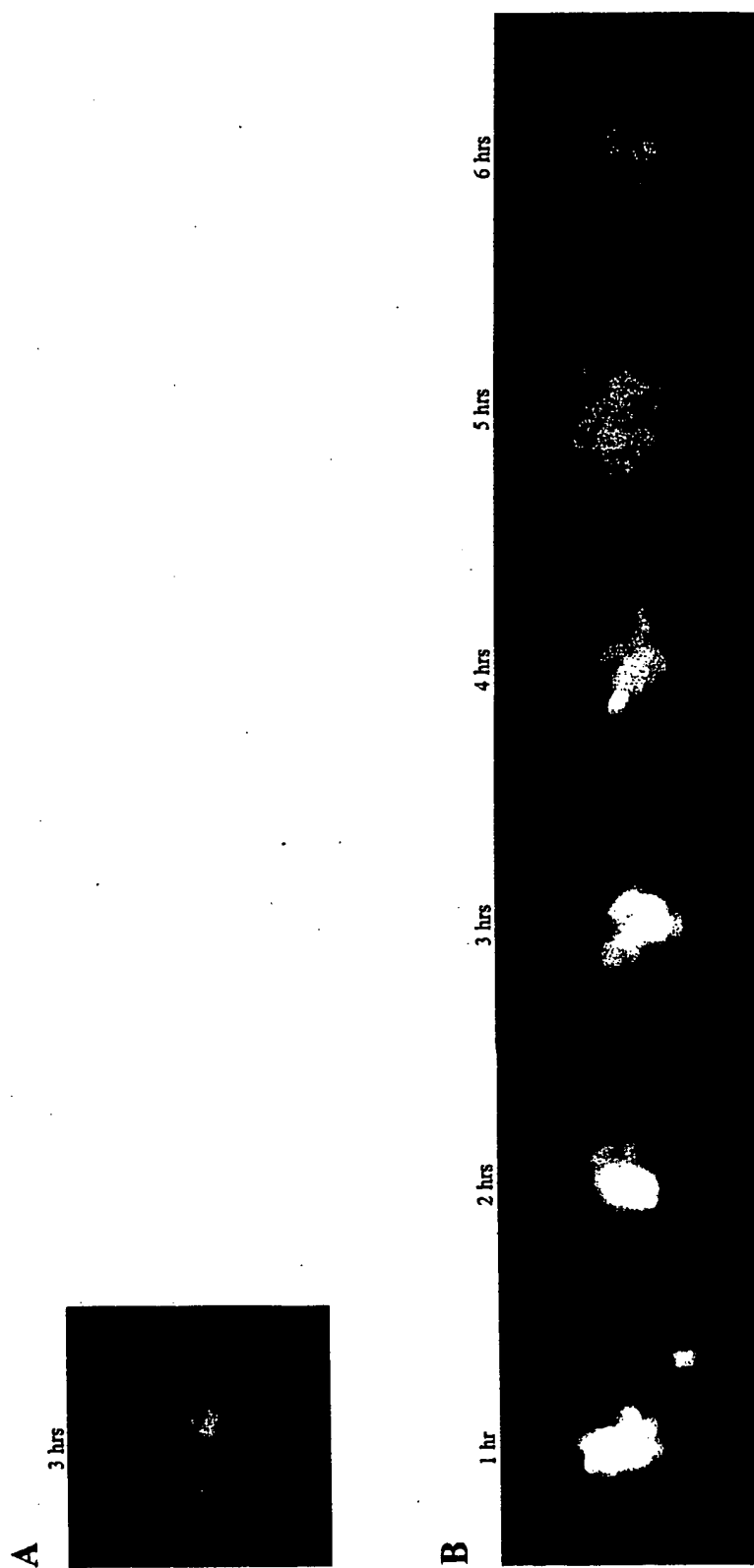
Fig. 8

A

B

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12Q1/68   G01N33/569   C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 497 464 A (AMOCO CORP) 5 August 1992	1,2,8-17
Y	see the whole document	4,5,18
X	WO 97 05282 A (UNIV GRONINGEN ;WELLING GJALT WIETZE (NL); SCHUT FREDERIK (NL); LA) 13 February 1997	1-3,6-17
Y	see the whole document	4,5,18, 23
X	WO 96 36734 A (ABBOTT LAB) 21 November 1996 see page 18; example 3	1,2,8-17
X	WO 96 34978 A (MACQUARIE RESEARCH LIMITED ;SYDNEY WATER CORP LIMITED (AU); VESEY) 7 November 1996 see page 7 - page 10	1,2,8-17

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[X] Further documents are listed in the continuation of box C.	[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1998	15/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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